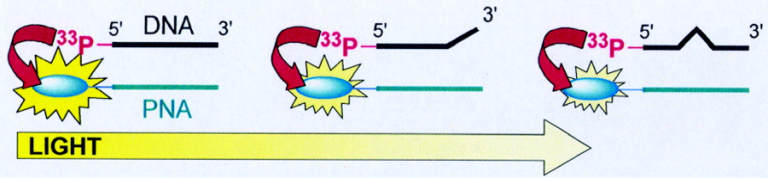
Report

A Platform for Both Solid-Phase Peptide Nucleic Acid Oligomer Synthesis and Subsequent in Situ Detection and Quantification of Nucleic Acid Sequences

Mark C. McCairn, Marcus D. Hughes, Anna V. Hine, and Andrew J. Sutherland

J. Comb. Chem., **2006**, 8 (5), 639-642• DOI: 10.1021/cc060035g • Publication Date (Web): 26 July 2006



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Journal of Combinatorial Chemistry is published by the American Chemical Society.
1155 Sixteenth Street N.W., Washington, DC 20036

A Platform for Both Solid-Phase Peptide Nucleic Acid Oligomer Synthesis and Subsequent in Situ Detection and Quantification of Nucleic Acid Sequences

Mark C. McCairn,[†] Marcus D. Hughes,[‡] Anna V. Hine,[‡] and Andrew J. Sutherland*

Department of Chemical Engineering & Applied Chemistry and School of Life & Health Sciences, Aston University, Aston Triangle, Birmingham, B4 7ET, United Kingdom

Received March 24, 2006

Introduction. A genetic disease may be identified by a mutation in the nucleic acid sequence of the associated gene.[1] The scintillation proximity assay (SPA) is a sensitive technique that can be used to detect and quantify DNA−DNA hybridization[2] and, thus, has the potential to detect such mutations. In a typical SPA, DNA is noncovalently attached to SPA beads (polymeric micro-spheres that encapsulate scintillant) and then screened against radiolabelled DNA.[3] Successful DNA−DNA hybridization brings the radiolabel into close proximity of the SPA bead, which elicits a scintillation signal that is proportional to the strength of the hybridization interaction.

Peptide nucleic acid (PNA) is a DNA mimic that can form a helical duplex structure with complementary single-stranded DNA (ssDNA) through Watson−Crick base pairing.[4] The strength of the interaction between complementary strands is stronger than in conventional dsDNA.[4] In addition, the *N*-(2-aminoethyl)glycine backbone of PNA is less susceptible to degradation by reagents and conditions commonly employed in solid-phase synthesis, as compared with the 2-deoxyribose phosphate backbone of DNA. Therefore, PNA oligomers may be synthesized readily upon an appropriately chemically functionalized, insoluble support from the corresponding PNA monomers using conventional solid-phase synthesis methodologies.[5] The insolubility of the support permits an excess molar amount of reagent(s) to be employed to drive reaction equilibria toward completion. In addition, supported PNA may be separated and purified simultaneously from both byproduct(s) and excess reagent(s) by simple filtration and washing procedures. Generally, the PNA is then cleaved from the support prior to screening in solution.[6] However, an on-support screening strategy is often more desirable, since it eliminates the requirement for additional synthesis and cleavage steps. Unfortunately, conventional SPA beads are incompatible with solid-phase synthesis because exposure of SPA beads to the majority of organic solvents dissolves the scintillant encapsulated in the pores of the SPA beads, which renders the beads useless for subsequent SPA.

Results and Discussion. Herein is reported for the first time a novel amphiphilic, chemically functionalized, scintillant-containing support, which was utilized in the efficient solid-phase synthesis of a number of PNA oligomers. The supported PNA oligomers were screened in situ against ^{33}P-ssDNA, which detected and quantified the extent of hybridization with number and position of PNA−DNA base mismatches with excellent sensitivity and selectivity.

Previously, we have reported the synthesis of a hydroxyl-functionalized, poly(oxyethylene glycol)-based, scintillant-containing support.[5] A related support of this type was required for use in the synthesis of a series of solid-phase PNA oligomers and subsequent in situ hybridization SPA. The support we had developed previously was deemed unsuitable for this application, since attachment of the initial *N*-protected fluorenylmethoxycarbonyl (Fmoc)-PNA monomer to a hydroxyl-functionalized support via an ester linkage is potentially susceptible to cleavage by monoketopiperazine formation upon removal of the Fmoc protecting group.[7] In addition, the penultimate step of the PNA oligomer library synthesis involves removal of benzhydryloxycarbonyl (Bhoc) protecting groups by treatment with trifluoroacetic acid (TFA), which most likely would cleave the ester linkage and, consequently, the PNA oligomer from the support. Therefore, we elected to construct a scintillant-containing, poly(oxyethylene glycol)-based, amine-functionalized (POPAM) support. The use of this support would result in the initial Fmoc-PNA monomer's being attached to the support via an amide linkage, which is far less susceptible than an ester linkage to cleavage by the reagents employed in the PNA oligomer synthesis.

Construction of a POPAM support with a controlled loading distributed homogeneously through the support matrix was enabled by synthesis of the intermediate monomer α-styryl-poly(oxyethylene glycol)$_{300}$ phthalimide **3**. α-Styryl-poly(oxyethylene glycol)$_{300}$ **1** was treated with a solution of toluenesulfonyl chloride (TsCl), triethylamine (TEA), and a catalytic amount of 4-*N*,*N*-dimethylaminopyridine (DMAP) in dichloromethane (DCM) to furnish the corresponding tosylate **2** (93%).[8] Purification of tosylate **2** was achieved by a facile washing procedure and subsequent employment of a nucleophilic scavenger resin. Tosylate **2** was then subjected to a two-step Ing−Manske modification of the Gabriel synthesis.[9] Initial reaction with potassium phthalimide in wet *N*,*N*-dimethylformamide (DMF) provided α-styryl-poly(oxyethylene glycol)$_{300}$ phthalimide **3** (76%) (Scheme 1).[10]

Unfortunately, hydrazinolysis of phthalimide **3** to the corresponding amine also reduced the styrenic double bond, which was evidenced by the disappearance of the signals corresponding to the styrenic double bond in the proton nuclear magnetic resonance spectra of the crude product. Therefore, α-styryl-poly(oxyethylene glycol)$_{300}$ phthalimide

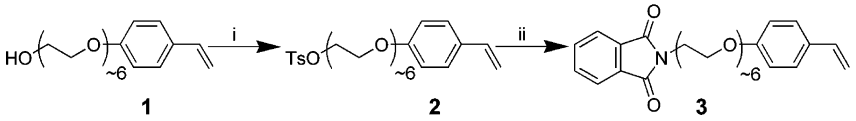
* Phone: +44 (0) 121 204 3425. Fax: +44 (0) 121 204 3679. E-mail: a.j.sutherland@aston.ac.uk.

[†] Department of Chemical Engineering & Applied Chemistry. Current address: School of Chemistry, University of Manchester, Oxford Road, Manchester, M13 9PL, United Kingdom.

[‡] School of Life & Health Sciences.

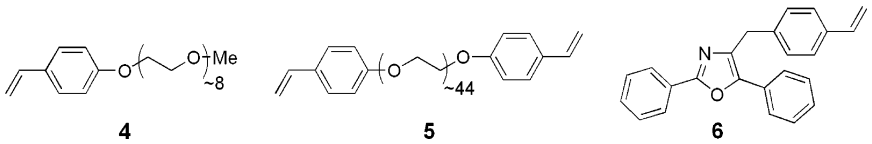
10.1021/cc060035g CCC: $33.50
Published on Web 07/26/2006

Scheme 1. Synthesis of α-Styryl-poly(oxyethylene glycol)$_{300}$ Phthalimide **3**[a]

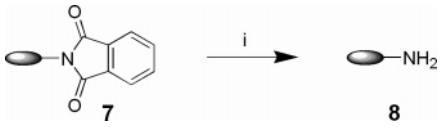


[a] (i) TsCl(1.2 equiv), TEA (2.4 equiv), DMAP (0.01 equiv), DCM, 0 °C − RT, 16 h, 93%; and (ii) potassium pthalimide (1.5 equiv), DMF/water (7.5:1), 80 °C, 16 h, 76%.

Chart 1. Structures of Monomers **4**−**6**



Scheme 2. Synthesis of POPAM Support **8**[a]



[a] (i) H_2N-NH_2 $(H_2O)_x$, dioxane, 50 °C, 16 h.

3 was first polymerized and subsequently hydrazinolyzed to generate the desired amine functionality.

The monomers α-styryl-poly(oxyethylene glycol)$_{350}$ monomethyl ether **4**, α,ω-bis-styryl-poly(oxyethylene glycol)$_{2000}$ **5**, and (4′-vinyl)-4-benzyl-2,5-diphenyloxazole **6** (Chart 1) were synthesized using a methodology that we have reported previously.[8,11] A monomer mixture containing **3** (6 mol percent), **4** (86 mol percent), **5** (2 mol percent), and **6** (6 mol percent) was combined with the free radical initiator 2,2′-azobisisobutyronitrile and subjected to a thermally initiated, radical, bulk polymerization reaction. This polymerization reaction proceeded smoothly to provide phthalimide-functionalized, scintillant-containing, poly(oxyethylene glycol)-based support **7** in the form of a gel, which was broken into small irregularly shaped particles (∼1 mm in diameter) using a spatula. The phthalimide groups of support **7** were converted into amine functional groups by treatment with a solution of hydrazine hydrate in dioxane to provide POPAM support **8** in quantitative yield (Scheme 2). The efficiency of the reaction was determined by infrared spectroscopy, which showed the complete disappearance of the imide C═O signal and the appearance of a strong, new NH_2 stretch.

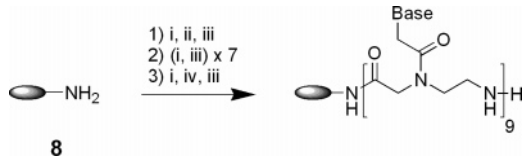
The compatibility of POPAM support **8** with a variety of solvents was determined by evaluating the percentage volume increase of **8** upon contact with solvent by utilizing a swelling assay procedure.[8,12] The percentage volume increase data obtained from this swelling assay established that **8** swells significantly with both aqueous solvent (water, 900%) and organic solvents (DCM, 1150%; tetrahydrofuran (THF), 900%; DMF, 900%; and toluene, 850%).

The accessible amine functionality of POPAM support **8** and the efficiency of a subsequent coupling reaction were determined by utilizing a standard Fmoc coupling/cleavage assay procedure.[13] Accordingly, two Fmoc-glycine residues were coupled sequentially to the amine functionality of **8** using conventional solid-phase chemistry. After each coupling reaction, the Fmoc-release assay was employed to determine the efficiency of each Fmoc-glycine coupling. The theoretical amine loading of **8**, based upon monomer composition, was calculated to be 0.11 mmol g^{-1}. After coupling the first Fmoc-glycine residue, the Fmoc-derived experimental loading of the support was 0.13 mmol g^{-1} (126%). This value suggests the more favorable polymerization of monomer **3** than one or more of the other monomers **4**, **5**, and **6** used in the construction of POPAM support **7**. The efficiency of the second Fmoc-glycine coupling reaction was observed to be quantitative, which suggests that POPAM support **8** is well-suited for use as a support in solid-phase PNA oligomer synthesis. This observation correlated with our previous evaluation of a hydroxyl-functionalized, poly(oxyethylene glycol)-based, scintillant-containing support.[5] This support, comprising the same polymer matrix as POPAM **8** and differing only in terms of the linker (OH instead of NH_2), was also rigorously assessed for its utility in the solid-phase synthesis of a PNA sequence ten residues long. In each case, an initial modest yielding coupling step of the first PNA residue, analogous to that observed for Fmoc-protected amino acids, was followed by successive coupling steps of Fmoc-PNA monomers, which all proceeded quantitatively. Since POPAM support **8** behaved in the same way as the hydroxyl-functionalized support in terms of coupling to Fmoc-protected amino acids, we elected to use it for the construction of a series of PNA oligomers without further evaluation.

A series of PNA oligomers were synthesized upon the amine functionality of POPAM support **8** for subsequent in situ screening against ^{33}P-ssDNA-A_9 in a hybridization SPA. Fmoc-PNA monomers (thymine, T; adenine, A) were coupled sequentially to the amine functionality of POPAM support **8** to generate a small number of supported PNA oligomers consisting of nine nitrogenous base residues of various sequence.

Experimentally, samples of POPAM support **8** were weighed accurately into a parallel array of separate reaction vessels. The activated ester of each Fmoc-PNA monomer was generated in situ by treatment with a solution of 1*H*-benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) and diisopropylethylamine in DMF. The activated ester of each Fmoc-PNA monomer was then added to the appropriate sample of POPAM support **8** and incubated. Unreacted amine functionality on the resultant Fmoc-PNA-derived POPAM support was capped by treatment with acetic anhydride. This coupling/capping procedure was followed by the selective cleavage of the Fmoc group

Scheme 3. Synthesis of POPAM-Supported PNA Oligomers[a]



[a] (i) Fmoc-PNA, PyBOP, $NEtPr^i_2$, DMF, 3 h; (ii) $(CH_3CO)_2O$, DMAP, pyridine, DMF, 2 h; (iii) 20% piperidine/DMF, 3 min; and (iv) 5% *m*-cresol/TFA, 1 h.

by treatment with piperidine. The coupling and deprotection procedure was repeated using the appropriate PNA monomers in succession. Finally, Bhoc protecting groups were removed by treatment with TFA, followed by cleavage of the terminal Fmoc groups to reveal the corresponding POPAM-supported PNA oligomers ($T_3T_3T_3$, $A_3T_3T_3$, $T_3T_3A_3$, $T_3A_3T_3$, T_4AT_4, $T_4A_2T_3$) (Scheme 3).

The POPAM-supported PNA oligomers were screened against ^{33}P-ssDNA-A_9 to determine the dependence of the number and position of complementary base pairs upon the strength of hybridization. A blank control was provided by screening POPAM support **8** against ^{33}P-ssDNA-A_9 to identify nonspecific interactions between ^{33}P-ssDNA-A_9 and the matrix of POPAM support **8**. A commercial sample of ssDNA-A_9 was 5′-radiolabelled with ^{33}P by treatment with [γ-^{33}P]-adenosine 5′-triphoshate ([γ-^{33}P]-ATP) and the enzyme T4 polynucleotide kinase (PNK).[14] Duplicate samples of the POPAM-supported PNA oligomer library and triplicate samples of POPAM support **8** were placed on sintered inserts, inside micro centrifuge tubes. To each sample was added an equal aliquot of ^{33}P-ssDNA-A_9 (2.0 pmol, 79426 cpm/pmol) in 6× SSPE buffer. After incubation, unbound material was separated from the support by centrifugation. Successively more stringent washes (4× SSPE, 2× SSPE, water, water 25 °C, water 30 °C, water 40 °C, and 3× 50% formamide/2× SSPE 40 °C) were employed by adding the wash solution to each sample on the sintered insert, centrifuging to remove the wash solution and unbound material, and then monitoring the support samples in a scintillation counter. The scintillation data obtained for the series of POPAM-supported PNA oligomers following the final wash with 50% formamide/2× SSPE at 40 °C is presented in Figure 1.

The scintillation data presented in Figure 1 suggests that ^{33}P-ssDNA-A_9 had annealed to the fully complementary POPAM-supported PNA oligomer $T_3T_3T_3$ to the greatest extent. This successful hybridization brought the ^{33}P-radiolabel into close proximity of the POPAM support, which elicited a scintillation signal (8036 cpm). Substitution of a single thymine codon (T_3) with a noncomplementary adenine codon (A_3) resulted in a significant reduction in the scintillation signal being detected. Substitution of the central thymine codon of POPAM-supported PNA oligomer ($T_3T_3T_3$) with an adenine codon ($T_3A_3T_3$) resulted in the greatest disruption of hybridization (505 cpm), followed by the 5′ terminus ($T_3T_3A_3$) (1622 cpm), whereas the codon closest to the POPAM support ($A_3T_3T_3$) caused the least disruption of hybridization (3805 cpm).

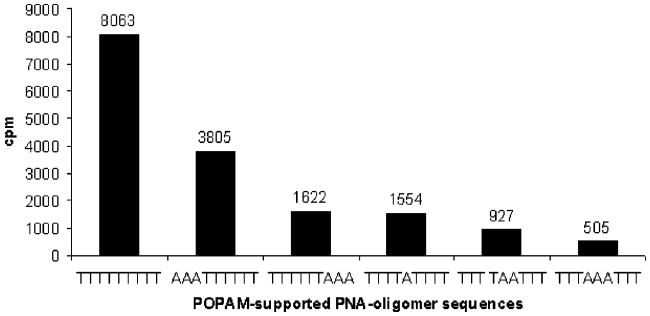


Figure 1. Scintillation counts per minute (cpm) detected for the hybridization assay of ^{33}P-ssDNA-A_9 against various sequences of POPAM-supported PNA oligomers. All scintillation data has been corrected by subtraction of the average number of scintillation counts (104 cpm) detected for the blank controls and then multiplied by 2.4 to accommodate the fact that support **8** scintillates with an efficiency of ∼41% compared with a multifluor scintillation cocktail.[5]

Having determined that substitution of the central thymine codon by an adenine codon caused the greatest disruption to PNA−DNA hybridization, the effect of substituting one (T_4AT_4) and two ($T_4A_2T_3$) thymine residues with adenine residues in the central codon was investigated. Substitution of the central thymine base residue in POPAM-supported $T_3T_3T_3$ with adenine (T_4AT_4) resulted in a significant reduction in the number of scintillation counts detected (1554 cpm), whereas substitution of a second thymine base residue with adenine ($T_4A_2T_3$) caused an additional 40% reduction in the scintillation signal detected (927 cpm). PNA−DNA duplexes are known to be more stable than the analogous DNA−DNA duplexes and are less susceptible to disruption by polar solvents.[15] However, even taking these factors into account, the fact that we observed PNA−DNA hybridization of such short sequences after such a number of high stringency washes is perhaps surprising but is consistent with our previous findings.[5]

Conclusions. A novel amphiphilic, amine-functionalized, scintillant-containing, poly(oxyethylene glycol)-based POPAM support **8** was constructed and utilized efficiently in the solid-phase synthesis of a number of PNA oligomers. The supported PNA oligomers were screened in situ against ^{33}P-ssDNA-A_9, which detected and quantified the hybridization dependence with the number and position of PNA−DNA base mismatches with excellent selectivity. In addition, the hybridization SPA was sufficiently sensitive to detect a single base mismatch successfully with a signal-to-noise ratio of 5:1. In previous work, we have observed signal-to-noise ratios of >300:1 when comparing a fully complementary PNA−DNA duplex of 10 residues in length with a totally noncomplementary PNA−DNA control experiment.[5] These signal-to-noise ratios compare favorably with those reported by Gaylord et al. in a fluorescence resonance energy transfer (FRET)-based study of a 14-base PNA probe sequence hybridizing to a longer-length ssDNA target which was ∼2:1 for fully complementary versus a single base mismatch and ∼30:1 for a fully complementary versus a totally noncomplementary sequence.[15a] It should be noted that when Gaylord et al. modified their assay procedure by the addition of S1 nuclease, significantly better signal-to-noise ratios were observed. It was reported that in this additional process,

PNA−DNA duplexes containing single base mismatches were digested upon exposure to the nuclease, whereas fully complementary duplexes were not similarly digested. In future work, it would be of interest to investigate the effect of adding S1 nuclease to the hybridization SPA and establishing if this process, in analogy with the elegant methodology developed by Gaylord et al., similarly enhances the signal-to-noise ratio of the SPA procedure.

We believe the SPA approach we describe herein should complement assay procedures based upon FRET. The SPA procedure should be more resistant to both chemical and photoinduced quenching than FRET-based applications. Moreover, in complex assay media, such as cell lysates, the signal-to-noise ratio should be higher, since the SPA will not be affected adversely by natural background fluorescence. For applications involving solid-supported synthesis in the presence of the reporter element, the chemical stability of the 2,5-diphenyloxazole scintillant reporter molecules is likely to be greater, upon exposure to many commonly encountered synthetic reagents, than the fluors commonly employed in fluorescent-based sensors. Accordingly, we anticipate that POPAM support **8** will find generic application in the high-throughput synthesis of combinatorial PNA oligomer libraries and subsequent in situ screening of these libraries against genetic disease targets of interest using the associated hybridization SPA.

Acknowledgment. We thank Aston University for financial support. We thank Dr. Mike Perry, Mrs. Karen Farrow, and Mr. Kevin Hughes (all of Aston University) for nuclear magnetic resonance spectroscopy, low-resolution mass spectroscopy, and scintillation counting studies, respectively, and Mr. Peter Ashton (Birmingham University) for high-resolution mass spectroscopy.

Supporting Information Available. Experimental procedures, assay procedures, and scintillation counting data. This information is available free of charge via the Internet at http://pubs.acs.org

References and Notes

(1) Collins, F. S.; Guyer, M. S.; Chakravarti, A. *Science* **1997**, *278*, 1580−1581.
(2) Schoenfeld, A.; Luqmani, Y. A. *Anal. Biochem.* **1995**, *228*, 164−167.
(3) Cook, N. D. *Drug Discovery Today* **1996**, *1*, 287−294.
(4) (a) Egholm, M.; Nielsen, P. E.; Buchardt, O.; Berg, R. H. *J. Am. Chem. Soc.* **1992**, *114*, 9677−9678. (b) Egholm, M.; Behrens, C.; Christensen, L.; Berg, R. H.; Nielsen, P. E.; Buchardt, O. *Chem. Commun.* **1993**, *9*, 800−801. (c) Nielsen, P. E.; Egholm, M.; Berg R. H.; Buchardt, O. *Science* **1991**, *254*, 1497−1500. (d) Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Norden, B.; Nielsen, P. E. *Nature* **1993**, *365*, 566−568.
(5) McCairn, M. C.; Hughes, M. D.; Hine, A. V.; Sutherland, A. J. *J. Comb. Chem.* **2006**, *8*, 1−3.
(6) Hyrupa, B.; Nielsen, P. E. *Bioorg. Med. Chem.* **1996**, *4*, 5−23.
(7) Nelson, K. E.; Levy, M.; Miller, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3868−3871.
(8) McCairn, M. C.; Tonge, S. R.; Sutherland, A. J. *J. Org. Chem.* **2002**, *67*, 4847−4855.
(9) (a) Ing, H. R.; Manske, R. H. F. *J. Chem. Soc.* **1926**, 2349. (b) Gabriel, S. *Chem. Ber.* **1887**, *20*, 2224.
(10) Gibson, M. S.; Bradshaw, R. W. *Angew. Chem. Int. Ed. Engl.* **1968**, 7, 919−930.
(11) Clapham, B.; Sutherland, A. J. *J. Org. Chem.* **2001**, *66*, 9033−9037.
(12) McCairn, M. C.; Hine, A. V.; Sutherland, A. J. *J. Mater. Chem.* **2003**, *13*, 225−231.
(13) *NovaBiochem Catalog and Peptide Synthesis Handbook*; NovaBiochem: La Jolla, CA, 1998; p S37.
(14) Sambrook, J.; Fritsch, E. F.; Maniatis, T. In *Molecular Cloning a Laboratory Manual 3*, 2nd ed.; Cold Spring Harbour: New York, 1989; p E. 19.
(15) (a) Gaylord, B. S.; Massie, M. R.; Feinstein, S. C.; Bazan, G. C. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 34−39. (b) Tomac, S.; Sarkar, M.; Ratilainen, T.; Wittung, P.; Nielsen, P. E.; Nordén, B.; Gräslund, A. *J. Am. Chem. Soc.* **1996**, *118*, 5544−5552.

CC060035G