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Report

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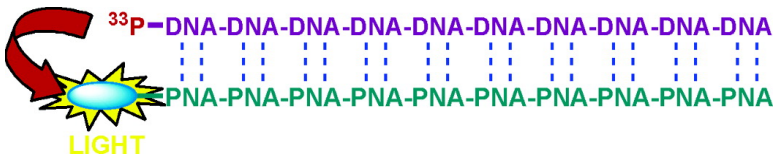

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Reports

Solid-Phase PNA Synthesis and In Situ Scintillation Proximity Assay for the Detection of PNA−DNA Hybridization

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Introduction. Drug discovery often involves the synthesis and subsequent screening of compounds against a biological receptor of interest. An efficient synthetic strategy employs solid-phase techniques whereby compounds are usually synthesized upon a chemically functionalized, insoluble, polystyrene-based support. The insolubility of the support permits an excess molar amount of reagent(s) to be employed to drive reaction equilibria toward completion. In addition, polymer-supported compounds may be separated and purified simultaneously from byproduct(s) and excess reagent(s) by simple filtration and washing procedures. These compounds are then generally screened for the ability to bind to a biological receptor target of interest after cleavage from the polystyrene-based support. An on-support screening strategy is often more desirable, since it eliminates the requirement for additional synthesis and cleavage steps. Unfortunately, the intrinsic hydrophobicity of polystyrene-based supports precludes their use in the aqueous environment of a biological screen.[1]

The scintillation proximity assay (SPA) is a powerful technique used to monitor receptor−ligand binding interactions in real time.[2] In a typical SPA, the receptor of interest is attached to SPA beads (polymeric microspheres that encapsulate the scintillant) and then screened against potential ligand molecules labeled with a suitable radioisotope. Successful receptor−ligand binding brings the radiolabel into close proximity of the SPA bead, eliciting a scintillation signal that is proportional to the strength of the binding interaction. Conventional SPA beads are incompatible with solid-phase synthesis; exposure of SPA beads to the majority of organic solvents dissolves the scintillant encapsulated in the pores of the SPA beads, rendering the beads useless for subsequent SPA.

Results and Discussion. Herein is reported for the first time a generic platform for both solid-phase synthesis and subsequent in situ SPA. To exemplify the utility of this strategy, a novel scintillant-containing polymer support was developed and used in the efficient synthesis of a peptide nucleic acid (PNA) oligomer using conventional solid-phase synthetic chemistry. This polymer-supported PNA oligomer was then used successfully in an in situ hybridization SPA against single-stranded DNA (ssDNA). Screening for complementary versus noncomplementary PNA−ssDNA hybridization revealed the excellent sensitivity of this novel assay procedure, which had a signal-to-noise ratio of over 300:1.

Initial work focused on constructing a chemically functionalized, scintillant-containing polymer that was both organic- and aqueous-solvent-compatible. Specifically, the poly(oxyethylene glycol) (PEG)-based monomers, α-styryl-poly(oxyethylene glycol)$_{350}$ monomethyl ether **1**, α-styryl-poly(oxyethylene glycol)$_{300}$ **2**, α,ω-bis-styryl-poly(oxyethylene glycol)$_{2000}$ **3**, and the scintillant monomer, (4′-vinyl)-4-benzyl-2,5-diphenyloxazole **4** (Chart 1), were synthesized using methodology that we have reported previously.[1,3] A monomer mixture that contained **1** (87 mol %), **2** (5 mol %), **3** (2 mol %), and **4** (6 mol %) was combined with the free radical initiator 2,2′-azobis-isobutyronitrile. This monomer mixture was subjected to a thermally initiated, radical, bulk polymerization reaction.[1] The polymerization reaction

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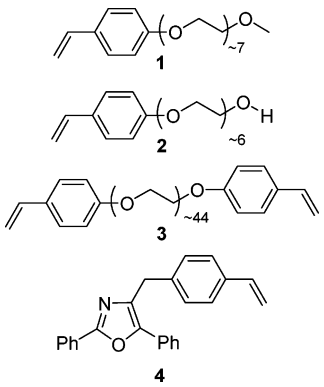
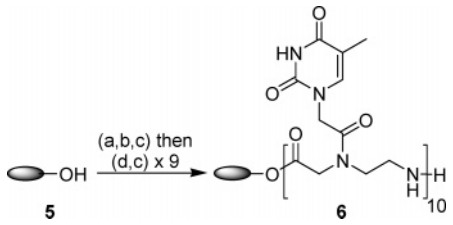
Chart 1. Structures of Monomers **1**, **2**, **3**, and **4**



Scheme 1. Synthesis of a Supported PNA−Oligomer 10 Thymine Units in Length, **6**[a]



[a] (a) 1-(Mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole (1 equiv), Fmoc-PNA-T (1 equiv), *N*-methylimidazole (3.7 equiv), DMF, RT, 2.5 h, 33%; (b) $(CH_3CO)_2O$ (1.5 equiv), 4-*N*,*N*-(dimethylamino)pyridine (0.05 equiv), pyridine (3.8 equiv), CH_2Cl_2, RT, 2 h; (c) piperidine (20% volume), DMF, RT; (d) Fmoc-PNA-T (2.7 equiv), 1*H*-benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (2.7 equiv), *N*,*N*-diisopropylethylamine (4.8 equiv), DMF, RT, 2 h, 100%.

proceeded smoothly to provide hydroxyl-functionalized, scintillant-containing, PEG-based support **5** in the form of small, irregularly shaped particles.

The compatibility of **5** with solvent was determined by evaluating the percentage volume increase of **5** upon contact with a variety of solvents by utilizing a syringe-based polymer-swelling assay.[1,4] The percentage volume increase data obtained from this swelling assay established that **5** is compatible with both aqueous (water, 650%) and organic solvents (dichloromethane, 1375%; tetrahydrofuran, 975%; *N*,*N*-dimethylformamide (DMF), 825%; toluene, 925%).

After determining that **5** has good aqueous and organic solvent compatibility, it was used to support the synthesis of a PNA oligomer (Scheme 1). N-Protected fluorenylmethoxycarbonyl-PNA-thymine (Fmoc-PNA-T) residues were coupled sequentially to the hydroxyl functionality of **5** using conventional solid-phase chemistry to produce a polymer-supported PNA oligomer of 10 thymine units, (PNA-T_{10}) **6**.[5] A standard Fmoc-release assay was employed to determine the efficiency of each Fmoc-PNA-T coupling reaction.[6] The theoretical hydroxyl-loading of **5**, based upon monomer composition, was calculated to be 0.09 mmol g^{-1}. After the coupling of the first Fmoc-PNA-T residue, the Fmoc-derived experimental loading of the polymer support was observed to be 0.03 mmol g^{-1} (33%). The efficiencies of subsequent Fmoc-PNA-T coupling reactions were observed to be quantitative.

Following the successful synthesis of **6**, we wished to test the in situ analysis capability of the support. Accordingly, a radiolabeled, single-stranded complementary sequence of DNA comprising 10 adenines (ssDNA-A_{10}) was prepared for use in a PNA−DNA hybridization assay. A commercial sample of ssDNA-A_{10} was 5′ radiolabeled with phosphorus-33 (^{33}P) by treatment with [γ-^{33}P]adenosine 5′-triphoshate ([γ-^{33}P]ATP) and the enzyme T4 polynucleotide kinase (PNK).[7] A second ssDNA oligomer, ssDNA-T_{10}, was similarly radiolabeled. Since ssDNA-T_{10} is not complementary to PNA-T_{10}, this sequence was used as a negative control in subsequent experiments to determine the extent of nonspecific interactions between ssDNA and the support. Finally, to account for the possibility of nonspecific interactions between the radiolabel ([γ-^{33}P]ATP) and **6**, a blank control was provided by combining all of the reagents in the absence of ssDNA.

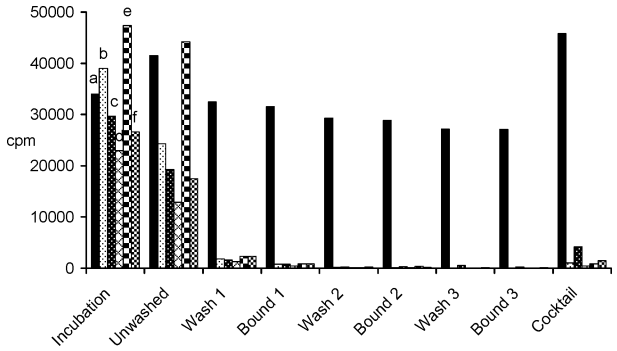


Figure 1. Scintillation counts per minute (cpm) detected for (a) **6**/^{33}P-ssDNA-A_{10}, (b) **6**/^{33}P-ssDNA-T_{10},[a] (c) **6**/blank control, (d) **5**/^{33}P-ssDNA-A_{10}, (e) **5**/^{33}P-ssDNA-T_{10},[a] (f) **5**/blank control with successive washing−counting/centrifugation−counting. All values obtained after addition of scintillation cocktail have been multiplied by 0.7 to accommodate the fact that a fluor based solely on 2,5-diphenyloxazole gives only 70% of the cpm detected with a multifluor scintillation cocktail.[9] (a) The value has been multiplied by 1.56 to accommodate the fact that ssDNA-T_{10} (2 766 442 cpm/pmol) was labeled with ^{33}P to a lesser extent than ssDNA-A_{10} (4 304 380 cpm/pmol), as determined by a standard DE81 assay.[10]

We elected to evaluate support **5** alongside **6** to assess potential nonspecific interactions with the matrix of the support. Accordingly, duplicate samples of **5** and **6** were placed on sintered inserts, inside micro centrifuge tubes. To these were added aliquots of ^{33}P-ssDNA-A_{10}, ^{33}P-ssDNA-T_{10}, and the blank control in 6×SSPE buffer. Each of the six assay mixtures was then monitored in a scintillation counter (Figure 1, Incubation). After incubation, unbound material was separated from the support by centrifugation, and the support samples were remonitored in a scintillation counter (Figure 1, Unwashed). Successively more stringent buffer washes (4×SSPE → 2×SSPE → water) were then performed by adding the buffer to the sintered insert, counting (Figure 1, Washes 1−3), centrifuging to remove the buffer, and again counting the support (Figure 1, Bound 1−3). Finally, each support sample was immersed in scintillation cocktail and monitored in a scintillation counter to determine the total bound counts (Figure 1, Cocktail).

The scintillation counting results presented in Figure 1 show that after resuspending the support samples in 4×SSPE buffer (Figure 1, Wash 1) a significant scintillation signal is only observed in the assay mixture that contained **6**/^{33}P-ssDNA-A_{10} (column a). Only very weak scintillation signals were observed for all of the control samples (columns b−f).

Moreover, an excellent signal (**6**/^{33}P-ssDNA-A_{10}, column a) to noise (**6**/^{33}P-ssDNA-T_{10}, column b) ratio of 18:1 (Figure 1, Wash 1) was increased significantly to 305:1 after two additional washing/centrifugation procedures (Figure 1, Bound 3). In addition, the support in the assay mixture containing **6**/^{33}P-ssDNA-A_{10} (Figure 1, Bound 3, column a) scintillated with excellent efficiency (59%) when compared with the residual number of counts detected after the addition of scintillation cocktail (Figure 1, Cocktail, column a).

Since it is well-known that PNA oligomers hybridize to complementary sequences of ssDNA,[8] these scintillation counting results suggest that ^{33}P-ssDNA-A_{10} has both permeated the matrix of the support and annealed to the complementary PNA-T_{10} of **6** successfully. This successful hybridization resulted in the ^{33}P-radiolabel being brought into close proximity of the support **6**, which in turn elicited a significant scintillation signal.

Conclusion. We have constructed a novel, scintillant-containing, PEG-based support **5** that is compatible with both aqueous and organic solvents. In a "proof of concept" study, **5** was used to support the efficient synthesis of a PNA oligomer 10 thymine units in length, **6**. The inherent scintillating capability of the support was subsequently exploited in an in situ hybridization SPA. This on-support screening procedure enabled the hybridization between PNA-T_{10} and its complement ssDNA-A_{10} to be detected efficiently and selectively over noncomplementary ssDNA-T_{10} and with an excellent signal-to-noise ratio. This factor coupled with a high degree of sensitivity means that the SPA approach we describe herein should complement assay procedures based upon fluorescence resonance energy transfer (FRET). The SPA procedure should be less prone to quenching (both chemical and photoinduced) than FRET-based applications, and in general, the signal-to-noise ratio should be higher, since the SPA will not be affected adversely by natural background fluorescence. In addition, the washing steps employed in the on-support screening procedure will also enable longer path length emitters, such as ^{14}C and ^{32}P, to be utilized as radiolabels in similar procedures. Therefore, we anticipate that amphiphilic supports of this type, with an in-built assay capability, will find generic application in the solid-phase synthesis and subsequent in situ analysis of polymer-supported entities such as PNA, DNA, peptides, and small organic compounds.

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

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