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ENHANCING SOLID PHASE SYNTHESIS BY A NONCOVALENT PROTECTION STRATEGY-EFFICIENT COUPLING OF RHODAMINE TO RESIN-BOUND PEPTIDE NUCLEIC ACIDS

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Abstract: Resins for solid-phase synthesis can affect coupling efficiencies by interacting with reactants. We have observed that polyethylene glycol-polystyrene (PEG-PS) solid support absorbs added activated fluorophores, preventing efficient labeling of peptide nucleic acids (PNAs). We now report that addition of an inexpensive unactivated fluorophore blocks the resin and allows efficient labeling. This protection strategy may have general benefits for peptide and combinatorial synthesis.

Introduction

Polyethylene glycol-polystyrene (PEG-PS) support possesses several advantages for solid-phase synthesis.[1] These advantages include improved mechanical strength and reduced swelling, making PEG-PS well suited for automated synthesis. We have used PEG-PS derivatized with either tri(alkoxy)benzylamide (PAL) or xanthen-2(or 3)-yl alanoic acid (XAL)[2] to synthesize peptide nucleic acid oligomers (PNAs)[3] (Figure 1a) of up to 30 bases as well as PNA-peptide chimera requiring up to 32 coupling steps.[4] Synthesis is efficient and PNAs can be purified to near homogeneity by C-18 reverse phase HPLC.

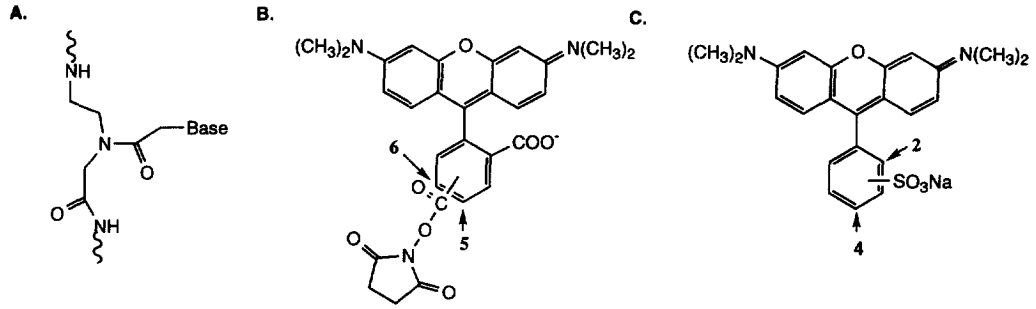

Figure 1. (a) Peptide nucleic acid. (b) Carboxytetramethylrhodamine succinimidyl ester (supplied as a mixture of isomers). (c) Sulforhodamine (supplied as a mixture of isomers).

Fluorophore-labeled PNAs are useful probes for identifying nucleic acid sequences by fluorescent in situ hybridization[5] and for monitoring cellular uptake of PNAs using fluorescent microscopy.[6] Conceptually, the attachment of fluorophores to PNAs should be easily accomplished by coupling the fluorophore to the N-terminal

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amine of the PNA while the oligomer remains on the resin. In our hands, however, we found that labeling required use of a large excess of carboxytetramethylrhodamine succinimidyl ester (Figure 1b), making the synthesis too expensive for routine use. Given the high efficiency of the coupling steps used to add PNA monomer, this inefficiency was surprising. A possible cause for poor labeling was revealed by visual examination of the resin, which became bright pink upon addition of rhodamine even when no PNA was present. We describe here an approach to protecting PAL or XAL solid supports that modifies its properties and allows efficient labeling of PNAs by rhodamine. This approach relies on noncovalent modification of the support and may be broadly applicable to peptide synthesis and combinatorial chemistry.

Results and Discussion

We synthesized PNA NH_2-Gly-CCCTAACCCTTACCCTAA-Lys-$CONH_2$ using XAL PEG-PS resin on a PE Biosystems Expedite 8909 automated synthesizer. The purpose of synthesis was to obtain fluorophore-labeled PNA for studies of cellular PNA uptake, so upon completion of the PNA the N-terminal Fmoc protecting group was removed and the succinimidyl ester of carboxytetramethylrhodamine (Figure 1b) was added in 12-fold excess over the estimated loading of PNA. After allowing the PNA to couple to fluorophore for 1 h we cleaved the PNA from the resin, removed the remaining protecting groups by treatment with TFA:*m*-cresol (4:1), and purified the PNA by reverse-phase C-18 HPLC. Surprisingly, mass spectral analysis revealed that unmodified PNA was the major product and that less than 10 % of purified PNA contained the fluorophore.

A standard solution to the problem of low yields during solid-phase synthesis is repetition of the inefficient coupling. Therefore we added the rhodamine ester in two steps with rhodamine present at a 12-fold excess during each coupling. Double coupling increased the efficiency of PNA labeling, but the added expense made it difficult for us to employ it as a routine procedure. An alternative procedure that uses less rhodamine ester is to cleave the PNA from the resin and then couple the rhodamine (in 5-fold excess) to a lysine or cysteine residue. We find that this procedure will generate fluorophore-labeled PNA in good yield and modest expense. Addition of a step that requires a solution-phase reaction, however, complicates purification of the labeled PNA because of the need to remove free rhodamine.

Examination of the PEG-PS support after treatment with rhodamine revealed that it was bright pink even after cleavage of the PNA from the resin. Addition of rhodamine ester to resin that had not been used for PNA synthesis also yielded bright pink support, indicating that rhodamine binds avidly to solid support regardless of the presence of PNA. We concluded that the capacity of the beads for binding rhodamine was high enough to affect the amount of fluorophore available for PNA modification and prevent efficient coupling.

We routinely use rhodamine labeling to monitor the cellular uptake of PNAs, and the low efficiency and high cost of PNA-fluorophore synthesis necessitated development of a strategy that would combine the advantages derived from use of PEG-PS support with improved labeling efficiency. We reasoned that it might be possible to block the resin with sulforhodamine (Figure 1c), a much less expensive reagent, thereby increasing the availability of carboxytetramethylrhodamine succinimidyl ester during the labeling step.

We examined two approaches to the noncovalent protection of PEG-PS resin. In the first approach, sulforhodamine was added to resin prior to initiation of synthesis. PNA monomers were then added sequentially without any modification of coupling protocols, and rhodamine ester was added at the end. In spite of repeated washing steps during automated synthesis the resin remained pink throughout the procedure. In our second

approach we first synthesized PNA using PEG-PS resin. We then treated the PNA-resin with sulforhodamine before removal of the N-terminal Fmoc protection. Subsequently, coupling with a 5-fold excess of the rhodamine ester was performed. Upon cleavage from the resin and deprotection, PNA that had been labeled by either method was analyzed. In both cases reverse-phase HPLC revealed two major products. The molecular weight of the two products was the same and was equivalent to that predicted for correctly synthesized PNA-rhodamine. The carboxytetramethylrhodamine ester is present in two isomers accounting for the separation of isomers that we observe. Yields of PNA (both complete and failed sequences) for both methods was ~59 % based on 2 μM estimated initial loading of the resin, with 85 % of this material being full length labeled product and the rest consisting of failed syntheses.

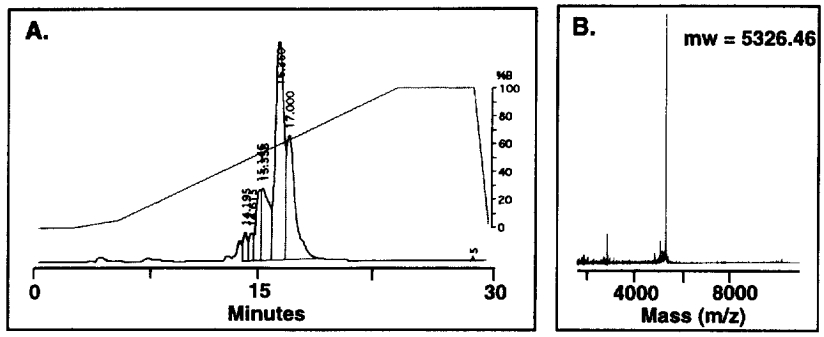

Figure 2. Labeling of PNA by carboxytetramethylrhodamine using resin that had been protected with sulforhodamine prior to synthesis. Labeling of PNA using XAL resin that was protect post synthesis produced identical results. (a) Purification of rhodamine labeled PNA by C-18 reverse-phase HPLC. (b) Mass spectral analysis (the calculated molecular weight was 5326.46).

Our two strategies have different strengths. The first strategy allows columns to be prepared in advance and would be useful for other solid phase synthesis that uses hydrophobic monomers during intermediate steps. Our second strategy leaves rhodamine addition for the final step, removing any possibility that its presence might interfere with subsequent coupling steps. Fluorophore-PNA conjugates generated by the two strategies have been delivered into cells and display identical properties compared to PNA-rhodamine synthesized using untreated PEG-PS support.

Conclusions

We have shown that noncovalent modification of PEG-PS support markedly influences the outcome of solid phase synthesis. Noncovalent modification is simple, and may have important applications beyond PNA synthesis to the use of hydrophobic reactants in peptide and combinatorial synthesis

Experimental

General. N,N-Dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Sigma-Aldrich (St. Louis MO). Diisopropylethylamine (DIEA) was obtained from PE Biosystems (Foster City, CA). Fmoc PNA monomers, activator [O-(7-aza-benzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate], and resin (PEG-PS XAL) were obtained from PE Biosystems. PNAs were synthesized on a PE Biosystems Expedite

8909 automated synthesizer and were cleaved from the solid support and deprotected using trifluoroacetic acid:*m*-cresol (4:1). Tetramethylrhodamine succinimidyl ester was obtained from Molecular Probes (Eugene OR). Sulforhodamine was obtained from Sigma-Aldrich. PNAs were purified by C-18 reverse phase HPLC as described[4] and were characterized by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DE workstation (PE Biosystems).

Labeling PNAs with rhodamine. To label PNAs without blocking the XAL PEG-PS resin, tetramethylrhodamine succinimidyl ester was dissolved to a concentration of 70 mM in 300 μL DIEA:DMF (1:30). This solution was introduced into a 1 mL syringe. This syringe and a second empty syringe were attached to a column packed with PEG-PS XAL resin containing the synthesized PNA. Using the two syringes, the solution was passed through the column for 60 min. The resin was then washed thoroughly with a 1:1 mixture of DMF and dichloromethane to remove uncoupled rhodamine. This procedure was then repeated to enhance coupling efficiency.

To improve labeling efficiency the XAL PEG-PS resin was treated with sulforhodamine prior to addition of rhodamine ester. Sulforhodamine was dissolved to a concentration of 70 mM in a 1:30 solution of DIEA:DMF. This solution (300 μL) was introduced into a 1 mL syringe. To modify the resin either before or after PNA synthesis, the syringe containing the sulforhodamine solution and a second empty syringe were attached to a column packed with XAL PEG-PS resin. Using the two syringes, the solution was passed over the column for twenty minutes. The resin was then washed thoroughly with a 1:1 mixture of DMF and dichloromethane to remove unbound sulforhodamine. Rhodamine ester is coupled to the N-terminus of the PNA by dissolving tetramethylrhodamine succinimydyl ester to a concentration 10 mM in 300 μL DIEA:DMF (1:30). Dual syringes were then used to pass the tetramethylrhodamine ester through the column as described above, followed by thorough washing with DMF. In each of the procedures described above, the N-terminal Fmoc protecting group was removed using piperidine prior to coupling of rhodamine ester to PNA.

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