Improved Conditions for Solid Phase Synthesis of Oligonucleotides on PS-PEG Copolymers

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Z. Naturforsch. 50b, 1096–1100 (1995); received January 20, 1995

PS-PEG Tentacle Polymers, Oligonucleotides, Solid Phase, ES-MS

Polystyrene-polyethylene glycol (PS-PEG) tentacle polymers with loadings of up to 60 µmol/g were used for standard oligonucleotide synthesis. As these resins are easy to handle and stable under reaction and cleavage conditions they may be used alternatively to controlled pore glass (CPG) as the most commonly used solid support for oligonucleotide synthesis. However, structural and chemical properties of the PS-PEG resins require modified conditions to guarantee syntheses with high coupling efficiencies. Oligonucleotides (ODN) of various sequences and lengths have successfully been synthesized using HPLC and capillary electrophoresis (CE) for purity control. Additionally, electrospray mass spectrometry (ES-MS) was used for product identification.

Introduction

The growing impact of gene technology provides an increasing demand for chemically synthesized ODN. The development of phosphoramidites as very reactive monomeric building blocks [1] together with the automatization of solid phase synthesis have made the fast and facile preparation of phosphodiester ODN feasible [2]. While these unmodified DNA fragments are widely used as PCR primers, units for gene synthesis or hybridization probes for genome analyses, functionalized ODN are increasingly applied to antisense technology to control gene expression and protein biosynthesis respectively [3].

Since the introduction of solid phase synthesis for ODN [4], a large number of solid supports was developed [5–11]. Due to the high synthesis yields CPG is the most commonly used resin yet [12, 13], though it can be affected by acid treatment during dimethoxytrityl (DMT) cation release or by basic deprotection at the end of synthesis [6, 14]. Furthermore, application of CPG supports is limited due to their low loading capacity which makes large scale synthesis or further functionalization of the support difficult and inefficient.

Coupling yields are often determined by DMT assay as the amount of released DMT cation is measured by absorbance or conductometry. This provides a facile possibility to monitor the current synthesis on-line. However, its systematical deviation factors make the DMT assay inappropriate for quantitative determination of synthesis yields [6]. Chromatographic (HPLC) and electrophoretic methods (CE), micellar electrokinetic capillary chromatography (MECC)) are, in contrast, well suited for reliable synthesis control [15–18]. Characterization of ODN can be carried out with ES-MS [19, 20], and even on-line coupling with HPLC is feasible with these substances yet [21].

In this report, the successful synthesis of ODN on PS-PEG copolymers are described while HPLC, MECC and ES-MS were used for purity control and product identification.

Materials and Methods

High performance liquid chromatography was carried out with a solvent delivery system S 1000 and low pressure gradient mixer S 8110 obtained from Sykam (Gilching, Germany) and a UV/VIS spectrometer UVI 204 from Linear (Reno, U.S.A.) using Nucleosil C18 and Capcell 8g 120 C8 reversed phase columns purchased from Grom (Herrenberg, Germany). Capillary electrophoresis was performed with the Grom capillary electrophoresis system 100 with an uncoated fused silica capillary and on-line detection at 260 nm with a Linear UV/VIS detector. For ES-MS analyses an
API III Taga 6000 E ionspray mass spectrometer obtained from Sciex (Toronto, Canada) was used. UV/VIS spectra for DMT assays were recorded on a Perkin-Elmer UV spectrometer Lambda 5. Additionally DMT cation release was automatically measured by conductometry at the synthesizer (DNA/RNA Synthesizer 394, Applied Biosystems).

PS-PEG copolymers (TentaGel S, TentaGel N) were obtained from Rapp Polymere (Tübingen, Germany). 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was purchased from Novabiochem (Bad Soden, Germany) and 4-methylmorpholine (NMM) from Fluka (Buchs, Switzerland). Protected deoxynucleoside succinates were obtained from Sigma Chemie (Deisenhofen, Germany). Dry solvents were stored over molecular sieves.

**Coupling of the nucleoside succinates on the PS-PEG supports**

Due to the required loading, the aminofunctionalized PS-PEG resins were suspended in dry DMF and combined with the corresponding equivalents of protected deoxynucleoside succinates, TBTU and NMM in a relation of 1:1:2. After 12 h of shaking the functionalized supports were filtered, washed with DMF, CH$_2$Cl$_2$ and methanol and dried under vacuum. While these coupling reactions are nearly quantitative no excess of succinate is needed. The resulting loadings were determined with DMT assay.

**Oligonucleotide synthesis**

Phosphorothioate and standard oligodeoxyribonucleotides were synthesized on Applied Biosystem DNA Synthesizer 380B and DNA/RNA Synthesizer 394 using phosphoramidite chemistry. All chemicals for solid phase synthesis were purchased from Applied Biosystems (Weiterstadt, Germany), Millipore (Eschborn, Germany) and Roth (Karlsruhe, Germany). Before synthesis on low capacity PS-PEG, unreacted amino groups of the support were capped with a slightly prolonged capping step using standard capping reagents. All further steps were carried out with modified conditions adapted for the synthesis on PS-PEG tentacle polymers (Table I).

**Results and Discussion**

PS-PEG tentacle polymers consist of polyethylene glycol chains which are grafted onto insoluble polystyrene matrices [24]. The PEG spacers have an average length of approximately 70 ethylene oxide units and a molecular mass of about 3000 dalton. Due to the excellent solvation properties and high mobility of PEG, a quasi-homogeneous system can be simulated [22]. Thus, the advantages of both, solid and liquid phase synthesis, are combined by this kind of support. With NMR relaxation time measurements and kinetic investigations, it has been shown that matrix effects are eliminated, as the PEG part dominates the solvation characteristics of the resin [23]. The amphiphilic behaviour of PEG guarantees high and constant swelling factors in polar (e.g. water, alcohol) as well as in aprotic unpolare solvents (e.g. CH$_2$Cl$_2$, DMF, acetonitrile).

However, there are quite decisive structural differences between PS-PEG and CPG supports which make changes in the synthesis conditions and protocols necessary. The kinetics and reaction times during synthesis on a PS-PEG support are mainly determined by diffusion constants of the reagents. In numerous syntheses we observed that slightly prolonged reaction times for coupling and oxidation steps resulted in better yields (Table I). PS-PEG copolymers are widely used for peptide synthesis [24]. For oligonucleotide chemistry, on account of their original high loadings of about

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagents</th>
<th>Time$^a$ [s]</th>
<th>Number of steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMT cleavage/wait</td>
<td>3% TCA$^b$ in CH$_2$Cl$_2$</td>
<td>8/15</td>
<td>6</td>
</tr>
<tr>
<td>Coupling/wait</td>
<td>0.1 M phosphoramidite in acetonitrile</td>
<td>2/15</td>
<td>1</td>
</tr>
<tr>
<td>Capping/wait</td>
<td>A: acetonhydride/lutidine/THF</td>
<td>1,5/15</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>B: 1-methylimidazole/THF</td>
<td>16/10</td>
<td>1</td>
</tr>
<tr>
<td>Oxidation/wait</td>
<td>0.1 M I$_2$/pyridine/H$_2$O/THF</td>
<td>8/30</td>
<td>1</td>
</tr>
<tr>
<td>Wash after oxidation</td>
<td>0.1 M ascorbic acid/pyridine</td>
<td>10/2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table I. Improved protocol for oligonucleotide synthesis on PS-PEG supports (ABI DNA/RNA Synthesizer 394).

$^a$ The first value refers to the reaction step, the second to the wait step; $^b$ trichloroacetic acid. All further steps are analogous to the standard protocols and thus not listed.
200–400 μmol/g, they were mainly applied to large scale synthesis using special synthesizers and reaction conditions [25, 26]. With high loaded PS-PEG supports, we observed average stepwise coupling yields (ASYs) of about 97%, even if excess of phosphoramidites was doubled. However, coupling efficiencies up to 97% are only acceptable for large scale syntheses of short oligomers. The preparation of longer ODN in the μmolar range requires ASYs of greater 98% to obtain efficient overall yields. On the other hand, in numerous continuous flow syntheses on high-loaded PS-PEG supports it could be observed that synthesis yields greatly depend on the sequence of the ODN. Oligomers containing a large number of thymidines were synthesized on PS-PEG resins with 200–400 μmol/g, they were mainly applied to large scale synthesis using special synthesizers and reaction conditions [25, 26]. With high loaded PS-PEG supports, we observed average stepwise coupling yields (ASYs) of about 97%, even if excess of phosphoramidites was doubled. However, coupling efficiencies up to 97% are only acceptable for large scale syntheses of short oligomers. The preparation of longer ODN in the μmolar range requires ASYs of greater 98% to obtain efficient overall yields. On the other hand, in numerous continuous flow syntheses on high-loaded PS-PEG supports it could be observed that synthesis yields greatly depend on the sequence of the ODN. Oligomers containing a large number of thymidines were synthesized on PS-PEG resins with

![Fig. 1. Sequence-dependent stepwise coupling deficit on high-loaded PS-PEG analyzed by MECC compared to DMT assay.](image1)

![Fig. 2. MECC separation of deca-nucleotides (5'CTGCA/TGCAT3') synthesized on PS-PEG resins with average loadings of a) 180 μmol/g, b) 100 μmol/g, c) 70 μmol/g and d) 40 μmol/g. The resulting ASY's were a) 97.1%, b) 97.9%, c) 97.8% and d) 98.3%.](image2)
loadings of about 200 \( \mu \text{mol/g} \) in excellent yields. In this way, an oligomer \( (T_8A_2T_4A_2T_8) \) was synthesized with an ASY of greater 99% on high-loaded PS-PEG (210 \( \mu \text{mol/g} \)). The sequence dependence of the stepwise coupling yields is demonstrated by comparison of four decanucleotides \( dT_{10} \), \( dC_{10} \), \( dA_{10} \) and \( dG_{10} \) (Fig. 1). As mentioned before, the average coupling deficits obtained from the assay by DMT cation release are always lower than those calculated from MECC measurements. To examine the influence of loading, mixed decanucleotides which could easily be separated from their failure sequences by MECC analyses were synthesized while the loading of the support was reduced stepwise from 180 to 40 \( \mu \text{mol/g} \). The ASYs were raised from 97.1% to 98.3% which means that the overall yields increased from 76.7% to 85.2% (Fig. 2).

In contrast to CPG with its hydrophilic and quite rigid surface, PS-PEG resins, due to their molecular composition and gel-like consistence, show strong interactions with some reagents used for ODN synthesis. The acid-induced release of the DMT cation for example was observed to proceed slower with increasing lengths of the ODN. Presumably the acid is buffered by a growing number of nucleobases as well as by the PEG chains of the resin. Thus, it is advisable to either use higher concentrated trichloroacetic acid or prolonged detritylation times for syntheses of longer ODN. After all, no depurination of the oligomer was observed using the modified synthesis conditions (Table I). Due to the strong interaction of the oxidation mixture (\( I_2, H_2O, THF, \text{pyridine} \)) with PS-PEG, most likely \( \pi \)-interactions between \( I_2 \) and the aromatic residues of polystyrene, acetonitrile is inappropriate as a washing solvent after the oxidation step. A solution of 0.1 M ascorbic acid in dry pyridine was used to purify the resin from oxidation reagents, whereas the excess of iodine was reduced and quantitatively removed.

Syntheses on the same PS-PEG supports with improved conditions in comparison to standard protocols always raised the ASYs for more than 3%. This underlines the importance of the reported changes of synthesis conditions. In Fig. 3a the HPLC profile of a crude mixed DMT-30mer synthesized on PS-PEG with an average loading of about 50 \( \mu \text{mol/g} \) under improved conditions is shown. Calculation of the ASY gave 98.6%. The crude product was characterized by mass spectrometry (ES-MS) with a mass deviation of 0.01% (Fig. 3b).

**Conclusion**

Using improved conditions and loadings up to 60 \( \mu \text{mol/g} \), PS-PEG tentacle polymers are excellent supports for standard synthesis of oligonucleotides in the \( \mu \text{molar scale}. The average stepwise coupling yields are all in the range of 98–99% and thus correspond with those received on CPG. The high loadings, available for PS-PEG copolymers, make facile and efficient functionalization of the support possible, even if high molecular residues for 3’-modification of ODN are re-
quired [27]. The functionalized resins can be obtained with loadings in a range of 50–100 μmol/g and are thus well suited for oligonucleotide synthesis.

HPLC, MECC and ES-MS are reliable analytical methods for purity control and characterization of oligonucleotides, as it is required for biological and pharmaceutical applications.

Acknowledgement

We gratefully acknowledge the support of this work from Applied Biosystems, Foster City, California by providing the synthesizer 394.