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Research Article

Synthesis and properties of radiolabeled CPTA–oligonucleotides

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Summary

A solid phase technique for the preparation of antisense oligodeoxynucleotides (ODNs) is described featuring 5′-end conjugated 4-[(1,4,8,11-tetraazacyclotetradec-1-yl)-methyl]benzoic acid (CPTA). Using Fmoc-protected CPTA–C6 amidite, CPTA was conjugated to ODNs at the end of an automated DNA synthesis. To illustrate successful conjugations, the CPTA–ODNs were labeled with ^{99m}Tc using the stannous-chloride reduction method. The resulting ^{99m}Tc complexes showed differences of stability between CPTA-conjugated and CPTA-unconjugated as well as 3′-protected and 3′-unprotected ODNs. Propane-1,3-diol 3′-modification enhanced efficiently the stability of ^{99m}Tc labeled ODN against exonuclease degradation. $Fmoc_3$CPTA-C6 amidite turned out to be a versatile ligand for radiometal complexation at the 5′-end. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: oligonucleotides; CPTA; solid phase synthesis; ^{99m}Tc

Introduction

Synthetic oligonucleotides have extensively been employed to suppress DNA transcription or RNA translation.[1] Thus, antisense oligonucleo-

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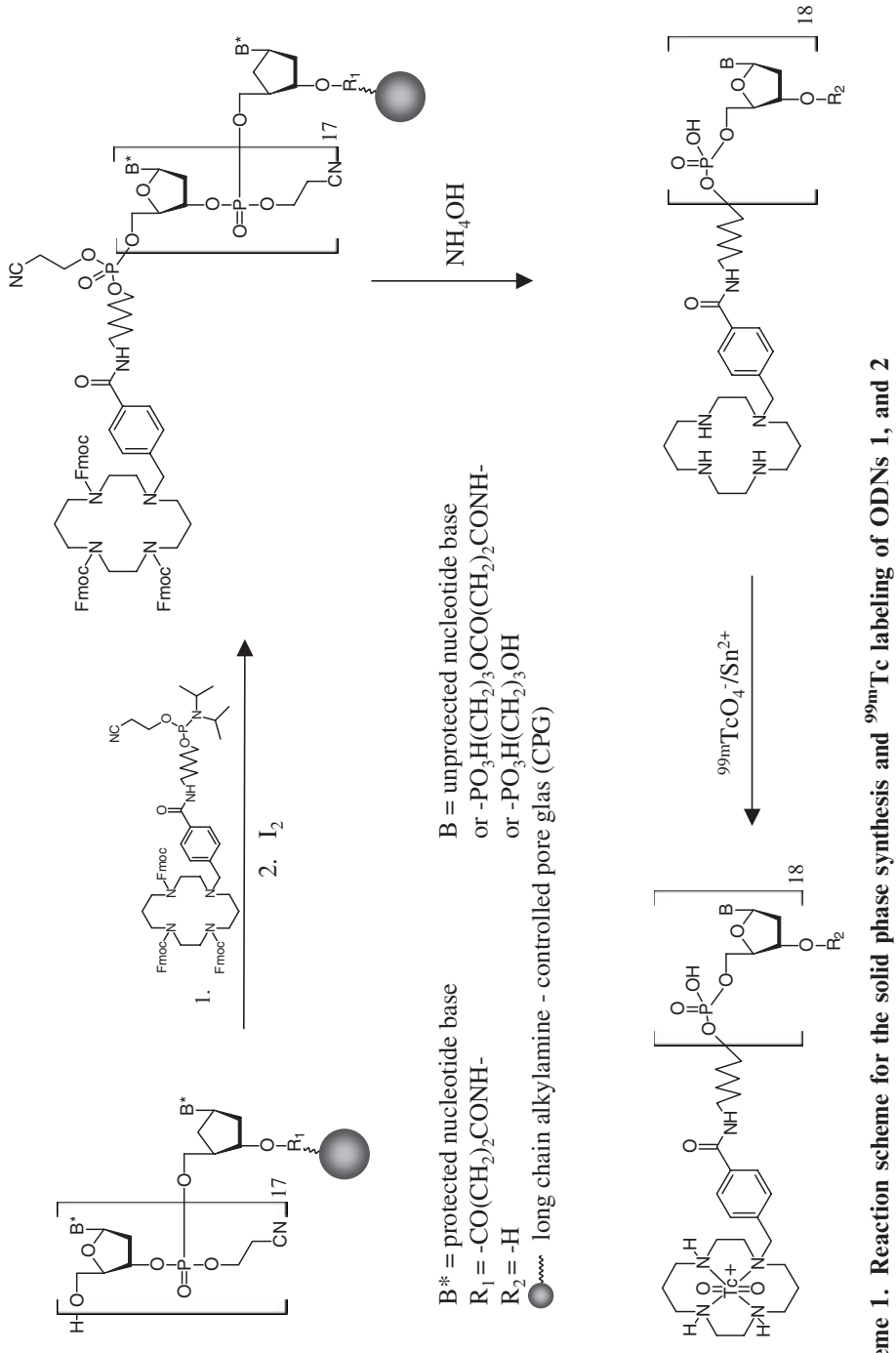
tides (ODNs) found a rapid transition from research tools to clinically administered drugs.[2,3] Because of the unique selectivity of hybrid formation with corresponding sense strands, ODNs are considered as attractive radionuclide carriers for molecular imaging. In addition, investigation of ODN metabolism and pharmacokinetics would be possible using radiolabeled ODNs.[4,5] For radiolabeling various ligand conjugations have been pursued including diethylenetriamine penta-acetate,[6,7] hydrazinonicotinamide,[8] mercaptoacetyltriglycine,[9] including ^{111}In and ^{99m}Tc complexation. Using *N*-(4-[^{18}F]fluorobenzyl)-bromoactetamide or the corresponding ^{76}Br derivative, PET tracers have successfully been introduced to 5′-thioate containing ODNs.[10–12] Other ODN labeling techniques using positron emitting radioisotopes also involved ^{76}Br[13] and ^{11}C.[14]

In order to extend the scope for PET and SPECT applications, a procedure for the automated synthesis of cyclam-ODNs conjugates was developed. Macrocyclic amines like cyclam form kinetically and thermodynamically stable complexes with a variety of transition metals especially ^{64}Cu[15] and ^{99m}Tc.[16–18] The aim of this study was to prove the versatility of 4-[(1,4,8,11-tetraazacyclotetradec-1-yl)methyl]benzoic acid (CPTA) conjugation to ODNs, to study complexation with ^{99m}Tc and to investigate the labeled CPTA–ODN conjugates for stability in aqueous solutions and human serum. In addition, the influence of 3′-end modification with 1,3-propanediol on the stability of ^{99m}Tc-CPTA conjugated ODNs was investigated.

Results and discussion

Solid-phase syntheses of modified deoxynucleotide and oligodeoxynucleotides

The syntheses of CPTA–C6-p5′TAACTACTGAGGTCACAA3′ (ODN 1), CPTA–C6-p5′TAACTACTGAGGTCACAA3′p-(propane-1,3-diol) (ODN 2) and 5′TAACTACTGAGGTCACAA3′ (ODN 3) were started using a standard DNA support (ODNs 1 and 3) and a 3′-modified propane-1,3-diol support (ODN 2), respectively.[19] As outlined in Scheme 1, the syntheses ended with the conjugation of $Fmoc_3$CPTA-C6 amidite at the 5′-terminal yielding ODNs 1 and 2. The chemistry of the CPTA conjugation was essentially based on standard phosphoramidite reactions which are generally used for oligodeoxynucleotide synthesis.



Scheme 1. Reaction scheme for the solid phase synthesis and ^{99m}Tc labeling of ODNs 1, and 2

At the end of the synthesis the modified ODNs were cleaved from the solid support using concentrated ammonia at 55°C for 5 h thereby releasing all protection groups. In departure from the standard procedure, addition of ethanol was necessary to ensure complete dissolution of the lipophilic Fmoc containing ODNs.

ODN purification was performed by RP-HPLC using ammonium-acetate/acetonitrile eluents. This system combines the advantage of high resolution and a volatile buffer system for product isolation. Analytical HPLC runs of CPTA–oligodeoxynucleotides (ODNs 1 and 2) and control (ODN 3) are shown in Figure 1. CPTA conjugation at the 5′-end (ODN 1) resulted in a 2.5 min increase of the retention time as compared to the control sample (ODN 3) even though one more negative charge was present from an additional phosphate group. 3′-End propane-1,3-diol-modification (ODN 2) led to a further increase of retention time.

ODNs 1, 2 and 3 were analyzed using MALDI-MS. As shown in Figure 2, the molecular mass of ODN 1 is in agreement with the calculated formula $C_{200}H_{266}N_{75}O_{107}P_{18}$: m/z = 5988 $[M+H]^+$; found: m/z = 5988 $[M+H]^+$. The peaks appearing at m/z = 6229 and at higher masses correspond to $[M+H]^+$ matrix adducts. ODN 2 showed a mass

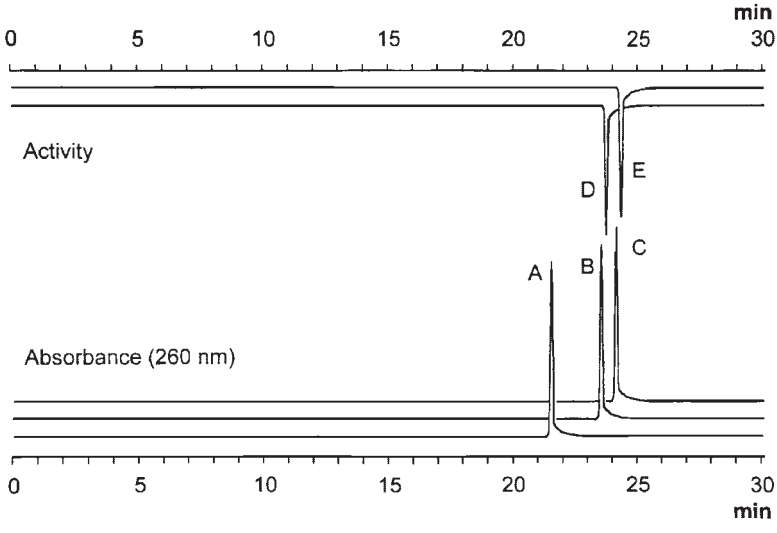


Figure 1. Reversed-phase HPLC of the unmodified ODN 3 (A), the CPTA–5′-modified ODN 1 (B) and the CPTA–5′/propane-1,3-diol-3′ modified ODN 2 (C). Radiochromatograms (top panel) depict ^{99m}Tc–ODN 1 (D) and ^{99m}Tc–ODN 2 (E)

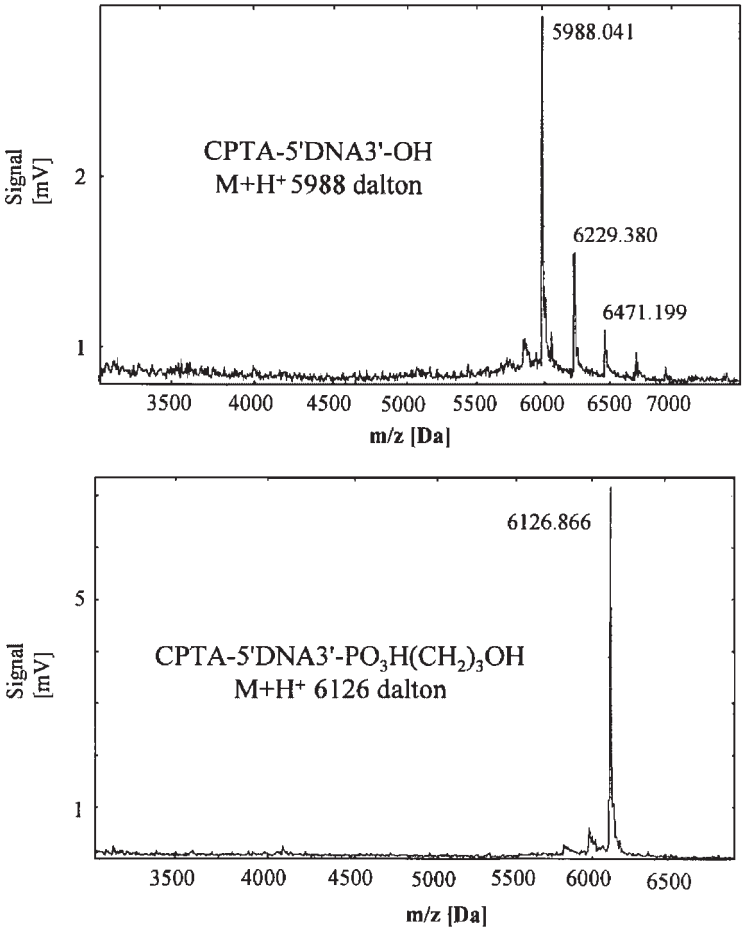


Figure 2. MALDI mass spectra of ODN 1 (upper) and of ODN 2

of m/z = 6126 $[M+H]^+$ which was in accordance with the calculated mass of m/z = 6126 ($C_{203}H_{273}N_{75}O_{111}P_{19}$ for $[M+H]^+$). The control ODN 3 revealed m/z = 5489 (calculated m/z = 5489 for $C_{176}H_{221}N_{70}O_{103}P_{17}$ $[M+H]^+$).

Radiolabeling of modified oligodeoxynucleotides

Prior to radiolabeling, ODNs 1 and 2 were heated at 70°C to disrupt secondary nucleic acid structures. Complexation of pertechnetate with CPTA modified ODNs was performed using stannous chloride. In order to facilitate ^{99m}Tc complexation with the strong base CPTA,

$pK_{H,1} = 10.63$,[20] reduction was performed at pH 11.5. Under these conditions the formation rate of $[^{99m}Tc(cpta)O_2]^+$ was reasonably fast yielding 45–60% labeled ODNs within 45 min. For comparison, CPTA alone afforded 79% radiochemical yield after 30 min.

Gel filtration was used to separate the labeled CPTA–oligonucleotide. RP-HPLC revealed a single radioactive peak of ^{99m}Tc labeled ODNs 1 and 2 showing an almost indistinguishable retention time for the unlabeled congener. Free $^{99m}TcO_4^-$ and ^{99m}Tc-tartrate complexes were completely retained on the size-exclusion column. The unconjugated ODN 3 was reacted as described for ODNs 1 and 2 yielding $<1\%$ of radioactivity associated with the oligonucleotide.

The specific activities of the ^{99m}Tc labeled ODNs 1 and 2 ranged between 12 and 16 GBq/μmol and were not optimized. This value may be enhanced to 30 GBq/μmol, thereby loosing, however, labeling yield. For imaging purposes the need for a higher specific activity might be met using HYNIC[8,21] as a ^{99m}Tc ligand. Likewise $[^{99m}Tc(CO)_3(H_2O)_3]^+$ in combination with histidine-based tags might be suitable for labeling ODNs.[22] In this study ^{99m}Tc served as a radiolabel to study the CPTA–ODN conjugate characteristics as a surrogate for ^{64}Cu which is one of the PET devoted radiometals for cyclam derived ligands.

Enzymatic digestion of ^{99m}Tc–ODN 1

In order to identify the site of ^{99m}Tc incorporation, ODN 1 was subjected to snake venom phosphodiesterase digestion. Integrals of the UV peaks appearing in the RP-HPLC of the enzyme digest were correlated with the extinction coefficients of the nucleosides present in ^{99m}Tc–ODN 1 (ε_{260}: dG 11700, dC 7300, dA 15400, dT 8800). The peak integrals were in accordance with the calculated nucleoside composition. The appearance of one radioactive peak coeluting with a UV signal not belonging to any of the four standard nucleosides indicated the presence of the ^{99m}Tc-CPTA tag.

In-vitro stability studies

In comparison to the control ODN 3, the stability of the CPTA-ODN conjugates ODNs 1 and 2 was studied at 37°C in aqueous buffer solutions between pH 5 and 9. The CPTA conjugates showed less degradation between pH 7 and 9 than at pH 5 indicating CPTA

protection against hydrolysis (Figure 3). The apparent half lives of the modified oligodeoxynucleotides in buffer at pH 7 were >12 days.

Exposure of the labeled CPTA–ODNs to human serum revealed a more pronounced difference in stability between ^{99m}Tc–ODN 1 and ^{99m}Tc–ODN 2. It is shown in Figure 4 that propane-1,3-diol incorporation at the 3′-terminal of ODN 2 improved the serum half life considerably. This finding is consistent with the known inability of

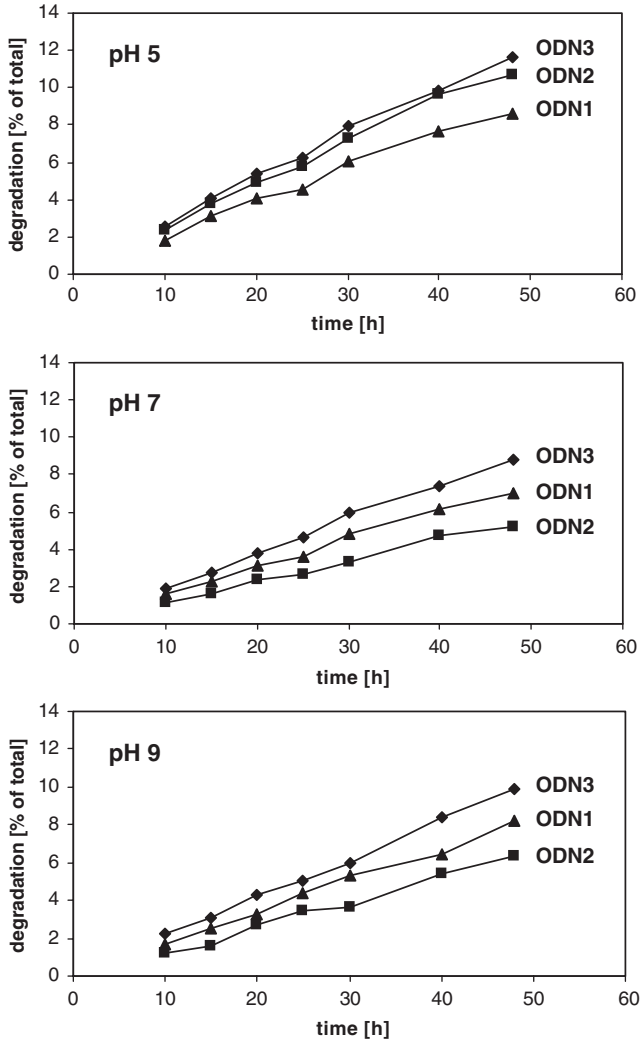


Figure 3. Stability of modified ODNs 1, 2 and unmodified ODN 3 in buffer solutions at pH values 5, 7 and 9. Values are means of triplicate measurements

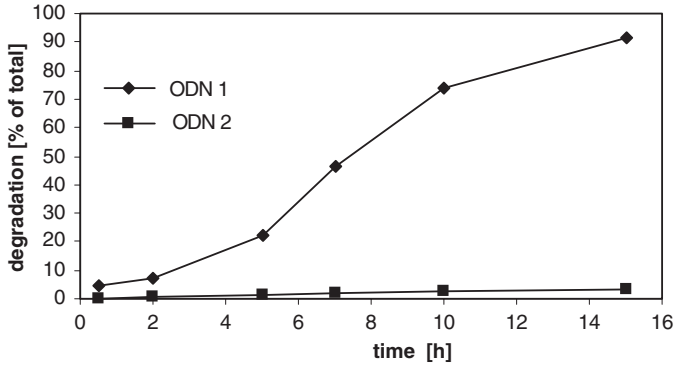


Figure 4. Stability of ^{99m}Tc–ODN 1 and ^{99m}Tc–ODN 2 in human serum at 37°C. Values are means of triplicate measurements

3′-exonucleases to recognize propane-1,3-diol 3′-protected ODNs as substrates.[19]

Experimental

All commercially available chemicals were of analytical grade and used without further purification. The modified nucleotide and oligodeoxynucleotides were assembled on a DNA synthesizer 380 B (Applied Biosystems, Foster City, CA) as described in the literature[23] using controlled pore glass (CPG) supports and appropriate protected nucleoside phosphoramidites (Applied Biosystems, Foster City, CA). Each synthesis cycle was performed on a 1.0 μmol scale. Gel filtrations were carried out with NAP-10 Sephadex columns (13 mm × 25 mm; Pharmacia, Uppsala, Sweden) using water as eluent. $Na^{99m}TcO_4$ was obtained from a $^{99}Mo/^{99m}Tc$ generator (DuPont, Germany). HPLC was carried out on Waters systems equipped with variable UV (Waters, Eschborn, Germany) and γ-radiation detectors (Radiomatic A 200, Canberra, Frankfurt, Germany). The reversed-phase HPLC columns used were PRP-1 C18 (10 μm, 305 mm × 7 mm, Hamilton, Switzerland). The solvent mixtures used throughout the experiments consisted of (A) 95% 0.1 M triethylammonium acetate at pH 6.5 with 5% acetonitrile (v/v), and (B) 30% 0.1 M triethylammonium acetate at pH 6.5 with 70% acetonitrile (v/v). The eluates were monitored at 260 nm. Electrospray ionization mass spectra (ESI-MS) were obtained on a Finnigan MAT TSQ 7000 injecting water or methanol solutions. Matrix assisted laser

desorption ionization mass spectrometry (MALDI-MS) was performed on a modified Bruker–Franzen (Bremen, Germany) REFLEX time-of-flight (TOF) mass spectrometer equipped with a gridless delayed extraction ion source and operated in the reflectron mode (matrix: 0.7 M 3-hydroxypicolinic acid and 0.07 M ammonium citrate in acetonitrile/water 1/1 (v/v)).

Solid-phase syntheses of CPTA-conjugated deoxynucleotide and oligodeoxynucleotides

11-[4-([6-*O*-[*O*-(2-Cyanoethyl)-*N*,*N*-diisopropylphospha-tidyl]oxy]hexyl-carbamoyl)-benzyl]-1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylic acid tris-(9*H*-fluoren-9-ylmethyl)ester ($Fmoc_3$CPTA-C6 amidite) was synthesized according to a procedure recently described.[23] The modified ODNs were synthesized on a DNA synthesizer in 1.0 μmol scale. The assembly of the modified ODNs was achieved using standard procedures[24] applying the following modifications: 1. 1.0 μmol loaded Dmtr-propane-1,3-diol-succinyl-CPG was employed as a support for the 3′-modification;[19] 2. for the 5′-modification 0.85 mmol $Fmoc_3$CPTA-C6 amidite was dissolved in 1 ml CH_2Cl_2/CH_3CN 1:3 (v/v), and placed into the unused uracil-reservoir of the DNA synthesizer. Additionally, the coupling time had to be increased to 720 s for complete coupling. The capping reaction with acetic anhydride and the removal of the dimethoxytrityl group with trichloroacetic acid were excluded from the last cycle of the automated synthesis.

The argon-dried support was fully deprotected within 5 h employing 0.1 ml ethanol/0.9 ml 32% aq. ammonia at 55°C.[23] Up to the penultimate step the coupling yield was greater than 90% as determined by colorimetric monitoring of Dmtr-cation release. The solutions of the ODN derivatives were filtered, lyophilized and purified by RP-HPLC with a linear gradient starting at 0% and ending at 18% B after 25 min.

^{99m}Tc complexation of CPTA-oligodeoxynucleotides

Prior to complexation, 200 μg CPTA-ODN derivative was heated in 30 μl water for 2 min at 70°C. After cooling to 40°C 300 μl $^{99m}TcO_4^-$ (900 MBq in 0.9% NaCl) was added within 15 min to the CPTA–ODN solution. Complexation was performed by adding 500 μl 150 μM Sn(II)-tartrate (adjusted to pH 11 with 0.1 M NaOH). The mixture was incubated for 1 h, and 300 μl aliquots were separated with NAP-10

Sephadex columns using water as a solvent. The labeled product was eluted within the fractions between 1 and 1.5 ml. The fractions were combined, lyophilized and subsequently purified by RP-HPLC, using a linear gradient starting at 0% B and ending at 18% B after 25 min. This process yielded pure ^{99m}Tc labeled ODNs 1 and 2. The unmodified control (ODN 3) was subjected to the same radiolabeling procedure. Specific activities obtained for ODNs 1 and 2 ranged between 12 and 16 GBq/μmol.

Enzymatic digestion of ^{99m}Tc-ODN 1

The position of the label in one of the modified oligodeoxynucleotides (ODN 1) was analyzed by enzyme digestion and HPLC. Thus, ^{99m}Tc–ODN 1 (20–40 μg) was incubated overnight at 37°C with 1 μl snake venom phosphodiesterase, 1 μl alkaline phosphatase (both Roche Diagnostics, Mannheim, Germany), 50 mM Tris HCl pH 8.0 and 10 mM $MgCl_2$ in a total volume of 20 μl. The resulting digestion products were analyzed by RP-HPLC using gradients from 0% B to 5% B in 5 min, to 6% B in 15 min, 6 50% B in 5 min and finally 50 to 80% B in 15 min.

In-vitro stability studies

The *in vitro* stability of the CPTA–ODNs was evaluated in buffer solutions at different pH. The incubations were performed at 37°C by mixing 60 μl 1 mM aqueous ODN solution with 0.5 ml of the following buffer solutions: pH 5, 50 mM citric acid, sodium phosphate buffer; pH 7, 50 mM Tris buffer; pH 9, 50 mM sodium carbonate buffer. Aliquots were removed at different time points and monitored at 260 nm using the RP-HPLC conditions described above.

To obtain an estimate of the stability of the radiolabeled CPTA–ODNs in the circulation, ^{99m}Tc-ODN 1 and ^{99m}Tc-ODN 2 were incubated in fresh human serum at 37°C for up to 12 h at a concentration of 30 μM unlabeled ODN. Control reactions containing only ^{99m}Tc-pertechnetate were run simultaneously under identical conditions. At different time points aliquots were centrifuged at 2500 g for 5 min in Centrisart I ultrafiltration tubes (Sartorius, Göttingen) with an exclusion limit of 30 kDa. The supernatants were analyzed by RP-HPLC measuring the radioactivity. The evaluation of these measurements took the following points into consideraton: the

purity of the sample, the fraction of ^{99m}Tc bound by the control and the amount of non-serum-protein bound radioactive constituents in the centrifuge residue.

Conclusion

CPTA–ODN conjugate synthesis was made feasible using $Fmoc_3$ CPTA–C6 amidite in a conventional DNA synthesizer. This method is to our opinion not restricted to phosphodiester ODNs. Phosphorothioates and mixed phosphorothioate/phosphodiester ODNs may also be used for the 5′-end CPTA modification. In addition to radiolabeling with ^{99m}Tc, complexation with positron emitting copper isotopes (e.g. ^{64}Cu) is likewise feasible.

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Abbreviations: CPG, controlled pore glass; CPTA, 4-[(1,4,8,11-tetraazacyclotetradec-1-yl)-methyl]benzoic acid; Dmtr, dimethoxytrityl; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; $Fmoc_3$ CPTA-C6 amidite, 11-[4-([6-*O*-[*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphatidyl]oxy]-hexylcarbamoyl)-benzyl]-1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylicacid tris-(9*H*-fluoren-9-yl-methyl)ester; MALDI, matrix assisted laser desorption ionization mass spectrometry; ODN, oligodeoxynucleotide; RP-HPLC, reversed-phase high-performance liquid chromatography.

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