Technetium-99m Labeling of DNA Oligonucleotides

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Single-stranded RNA and DNA oligonucleotides may be useful as radiopharmaceuticals for antisense and other in vivo applications if convenient methods for stably attaching radionuclides such as ^{99m}Tc can be developed. **Methods:** To radiolabel DNA with ^{99m}Tc, we have used the hydrazino nicotinamide (SHNH) moiety developed elsewhere. The diethylenetriaminepentaacetic acid (DTPA) chelate was used to label DNA with ^{111}In for comparison. Complementary 22-base, single-stranded oligonucleotides were obtained, each with a primary amine attached to either the 3' or 5' end and with a biotin moiety on the opposite end. The DNA was conjugated with SHNH by a N-hydroxysuccinimide derivative and with DTPA by the cyclic anhydride. **Results:** Reversed-phase HPLC analysis showed that essentially complete conjugation was achieved in both cases. The purified SHNH-DNA was radiolabeled with ^{99m}Tc by transchelation from glucoheptonate at labeling efficiencies of up to 60% and DTPA-DNA with ^{111}In acetate at up to 100% efficiency. After labeling, the ability of the DNAs to bind to streptavidin through the biotin moieties and to hybridize with their complementary DNA in saline was retained for both radiolabels as determined by size-exclusion HPLC analysis. HPLC radiochromatograms of serum incubates showed a shift of ^{99m}Tc, but not ^{111}In, to a high molecular weight, strongly suggesting serum protein binding in the former case only. Low-molecular weight degradation products were seen with ^{111}In, but not with ^{99m}Tc and may be related to the use of phosphodiester-linked oligonucleotides. As a further measure of label stability, the DNAs were bound to streptavidin-conjugated magnetic beads and incubated in fresh 37°C human serum. Less than 4% of ^{99m}Tc and 14% of ^{111}In was lost in 24 hr. **Conclusion:** Amino-modified, single-stranded DNA can be stably radiolabeled with ^{99m}Tc by the SHNH moiety without loss of function.

Key Words: oligonucleotides; technetium-99m; radiolabeling

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$\mathbf{T}$he extraordinary properties of DNA and RNA suggest that there is potential for the use of these oligonucleotides as radiopharmaceuticals. For example, there is current interest in antisense applications in which oligonucleotides

are used, either to block transcription of genes within the nucleus of cells or to block translation of messenger RNA within the cytoplasm (1,2). Accordingly, if radiolabeled, these oligonucleotides may usefully carry radioactivity to targeted cells or tissues. This and other applications of these reagents as radiopharmaceuticals, however, will require that methods be developed for radiolabeling them with diagnostic and, possibly, therapeutic radionuclides. Methods for radiolabeling oligonucleotides with beta-emitting radionuclides such as ^{3}H, ^{35}S and ^{32}P are well established (3–6); however, the use of oligonucleotides radiolabeled with imageable radionuclides is in its infancy. Methods have been reported for labeling oligonucleotides with ^{125}I (6,7) and, as such, these methods should be useful with ^{123}I and ^{131}I radionuclides with imaging properties. Depending on the pharmacokinetic properties of an oligonucleotide of interest, it is likely that the imaging radionuclide of choice will often be ^{99m}Tc. Recently, a method for radiolabeling DNA with ^{99m}Tc was described which relies upon a derivative of DTPA attached to the oligonucleotide to form chelates with reduced ^{99m}Tc (8). DTPA has previously been used for radiolabeling antibodies with ^{99m}Tc but was abandoned when the instability of the label was judged to be unacceptably high (9). Accordingly, this study was performed to investigate an alternative approach to label oligonucleotides stably with ^{99m}Tc. A hydrazino nicotinamide (SHNH) moiety has been shown to form stable complexes with ^{99m}Tc when conjugated to antibodies (10). In this investigation, an amine-derivatized DNA was conjugated with SHNH and the properties of the label evaluated. To provide a useful comparison, the same DNA was also conjugated with DTPA, as has been described by others (2,11), for radiolabeling with ^{111}In using procedures routine for antibody labeling (12).

In this investigation, 22-base, single-stranded DNAs were selected because their small size (about 8 kDa) was expected to facilitate rapid whole-body clearance after administration. Moreover, a 22-base oligonucleotide is large enough to virtually exclude any possibility of an accidental match within the genomic DNA and its transcripts (1). DNAs used were derivatized on one end with a biotin moiety to provide a useful means of establishing labeling efficiency and label stability. Using this feature, radiolabeled DNAs may be easily distinguished from unbound or
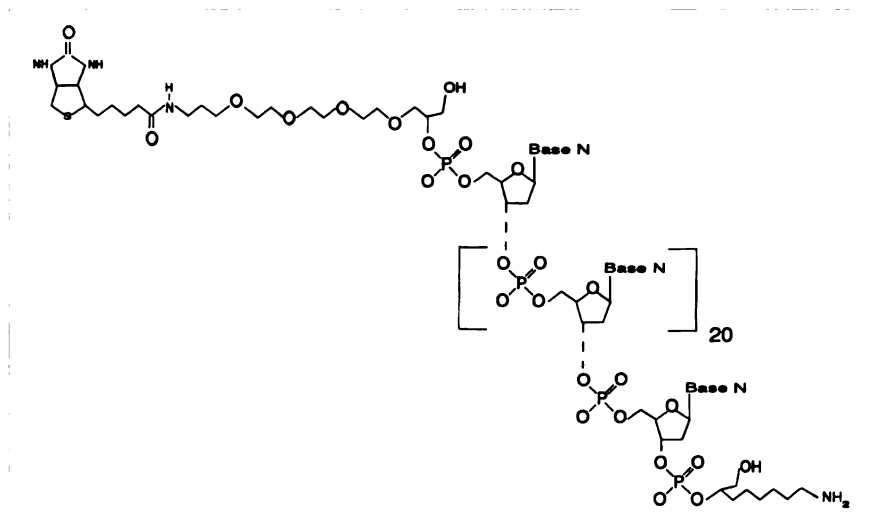
dissociated label by size-exclusion analysis before and after the addition of streptavidin (13).

In this study, we describe the results of conjugating several single-stranded DNAs with SHNH and DTPA followed by labeling with ^{99m}Tc and ^{111}In, respectively. The stability of the label was determined in serum, and biodistribution studies were performed in normal mice.

MATERIALS AND METHODS

Four 22-base, single-stranded DNA sequences were purchased (Operon Technologies, Alameda, CA) for this investigation. The base sequences were 5'-biotinTA ATA CGA CTC ACT ATA GGG AGamine-3' (A-chain) and 5'-amineGG TAC AGG TCT CAC TGT ATG ACbiotin (C-chain) and their complements (B- and D-chains, respectively). Figure 1 presents the structure of the A- or B-chain (i.e., with the biotin moiety on the 5' end). As shown, the biotin moiety was attached directly through a 15-member amide-polyether linker to the terminal phosphate, while the amine was normally attached to the terminal phosphate group through a 6-member methylene carbon spacer. The molecular weight of each chain was about 8.2 kDa. The melting and annealing temperatures in physiological saline were calculated to be 57–60°C and 32–41°C, respectively, for both pairs (14). The DNAs were purchased unpurified and were used without further purification. They were generally handled under sterile conditions; all solutions were sterilized by terminal filtration through a 0.22-μm filter, and sterile pipette tips were used. All other pipette tips and tubes were autoclaved prior to use.

Streptavidin was purchased and used without further purification. The DNAs were stored dry as received, at refrigerator temperatures and were dissolved at a concentration of 1–4 mg/ml in sterile water when needed. After solubilization, 20–500 μg DNA were added to sterile plastic vials which were immediately frozen for storage. Technetium-99m-pertechnetate was obtained from a ^{99}Mo-^{99m}Tc radionuclide generator and ^{111}In was purchased as the chloride complex. Streptavidin-conjugated superparamagnetic polystyrene beads (Dynabeads M-280, Dynal, A.S., Lake Success, NY) were stored at refrigerator temperatures as recommended by the manufacturer. The N-hydroxysuccinimide (NHS) derivative of the hydrazino nicotinamide moiety was a gift from Dr. M. Abrams,

(Johnson Matthey Inc., West Chester, PA). The cyclic anhydride of DTPA was synthesized as previously described (15).

Oligonucleotide Conjugation

In this investigation, all four DNA chains were derivatized with either SHNH or DTPA. In the former case, the NHS-derivative of SHNH moiety was dissolved in dry dimethylformamide to a concentration of 8.6 mg/ml (10). For conjugation, the DNA solution was diluted with a sterile bicarbonate buffer so that the final concentrations were 2.0 mg/ml DNA, 1 M NaCl, 0.25 M NaHCO$_3$, 1.0 mM EDTA at pH 8.3–9.0. The EDTA was added to complex cations such as calcium. The DNA solutions were incubated for 30 min at 45°C just prior to SHNH addition to dissociate any secondary complexes between the primary amines and the phosphate backbone. Between 0.2 to 1 mg of DNA was conjugated at a 1–25 molar excess of SHNH to DNA by adding the necessary volume of dimethylformamide solution dropwise to the DNA solution while vortexing. The solution was incubated at room temperature for 1 hr. Following incubation, the conjugated oligonucleotide was purified on a 0.7 × 20 cm gel filtration column of P-4 using a sterile 0.25 M ammonium acetate, 1.0 mM DTPA buffer at pH 5.2 as eluant. The DTPA was added to complex excess stannous ion and help prevent radiocolloid formation. Fractions (0.4 ml) were collected and the absorbance (260 nm) of each measured. Oligonucleotide concentrations were estimated using an extinction coefficient determined in this laboratory of 30 μl/μg for a 0.1% solution measured at 260 nm. The absorbance of SHNH at this wavelength and under these conditions was found to be insignificant. The fractions collected contained DNA at concentrations in the 0.5–1.0 mg/ml range.

Conjugation with DTPA was achieved with the cyclic anhydride as previously described for proteins (12). Approximately 1 mg of the oligonucleotide was dissolved in 10 ml 0.4 M HEPES buffer, pH 8.5, and this solution was added rapidly to a round-bottom test tube containing 100 mg of the dry anhydride while vortexing. Thus, the DTPA:DNA molar ratio was 2110:1. The solution was incubated at room temperature for 30 min. The conjugated oligonucleotides were originally purified on a gel-filtration column of P-2 using 10% ethanol as an eluant, but the inability to remove an unidentified radiochemical contaminant using this column necessitated the use of an alternative purification method. The reaction

mixture was adjusted to pH 3.5 using glacial acetic acid and was purified on a 1.7 × 9-cm column of DEAE Sephadex in the acetate form (Sigma) by stepped elution. The first 50 ml of eluant were 1% acetic acid. The next 90 ml of eluant were 1% ammonium acetate, pH 5.5, in which the concentration of NaCl was increased from 0.0 to 0.5 M in five equally spaced steps. The final eluant consisted of 20 ml 1% acetic acid, 0.5 M NaCl. The oligonucleotides were eluted between 139–153 ml as determined by absorbance at 260 nm and by polyacrylamide gel electrophoresis. The peak fractions were pooled, aliquoted into small fractions and stored frozen.

The extent of both SHNH and DTPA conjugation to oligonucleotides was determined by reversed-phase high performance liquid chromatography (HPLC) using a 4 × 250-mm C-18 column (BioRad), a flow rate of 0.8 ml/min and a linear gradient over 40 min from 0.07 M NaClO$_4$ to 100% acetonitrile. Unconjugated oligonucleotides elute with a retention time of about 12 min, while the SHNH-conjugated oligonucleotide elutes at about 21 min. Free DTPA elutes at or near the void while the DTPA-conjugated oligonucleotide elutes at about 3 min.

Oligonucleotide Labeling

The SHNH-conjugated oligonucleotides were radiolabeled by transchelation from [99mTc]-glucoheptonate as has been previously described for the labeling of mercaptoethanol-reduced antibodies with [99mTc] (16). Several vials were prepared under nitrogen, each containing 200 mg glucoheptonic acid and 140 μg SnCl$_2$ · 2H$_2$O at a final pH of 5.5 and were lyophylized. For reconstitution, 3 ml nitrogen-purged saline was added through the vial septum. Prior to radiolabeling, the SHNH-conjugated oligonucleotide solution after P-4 purification was incubated for 10–15 min at 45°C to dissociate any secondary complexes. Between 5–10 μCi [99mTc]pertechnetate eluant was added to each microgram of conjugated oligonucleotide. Immediately thereafter, 20 μl of the reconstituted glucoheptonate kit was added (i.e., 1.3 mg of glucoheptonic acid and 1.0 μg of SnCl$_2$ · 2H$_2$O) for [99mTc] activities up to 1 mCi. The solution was incubated for 1 hr at 45°C. The labeled oligonucleotide was then purified on a 0.7 × 20-cm gel filtration column of Sephadex G-50 using sterile saline as eluant. Radioactivity as well as absorbance at 260 nm was used to identify and quantitate peak fractions. As a control, the identical labeling procedure was applied to the unconjugated oligonucleotide.

Late in this investigation, glucoheptonate was replaced with tricine as transchelator (17). A fresh sterile solution of tricine (Sigma) in 0.02 M NaOH, pH 7.0, at a concentration of 70 mg/ml was prepared and mixed with a fresh solution of stannous ion in 0.1 M HCl so that 1.4 μl of the tin solution was added to each 150 μl of the tricine solution. Typically, an aliquot of this solution would be added to a solution of pertechnetate and SHNH-conjugated DNA so that the final concentrations were 18 mCi/ml [99mTc]pertechenetate, 0.05 mM DNA, 0.14 M tricine, 0.15 mM SnCl$_2$ · 2H$_2$O, pH 7. The labeling solution was incubated at room temperature for 10 min before purification by G-50.

The DTPA-conjugated DNA was radiolabeled after DEAE purification with [111In]-labeled acetate, as is routine for labeling DTPA-coupled antibodies with this radionuclide (12). The acetate complex was prepared by adding 1 M sodium acetate, pH 6, to the acidic [111In]-chloride solution. Up to 180 μCi [111In] were added to 1.6 μg DNA. The labeled DNA was purified on a 0.6 × 20-cm gel filtration column of Sephadex G-25 using sterile saline as eluant. Radioactivity as well as absorbance at 260 nm was used to identify and quantitate peak fractions. As a control, the identical labeling procedure was applied to the unconjugated oligonucleotide.

Electrophoresis

A denaturing 20% polyacrylamide gel was used for the analysis of DNA with a 89 mM Tris borate, 2 mM EDTA buffer at pH 8.3 (TBE) (18). Samples for analysis were prepared in TBE containing 40% formamide and 0.05% each of bromophenol blue and xylene cyanole. The solution was heated at 80°C for 10 min prior to loading on the gel. Each lane contained 100–400 ng DNA added in 1–5 μl and the electrophoresis was performed at 12 mA under constant water cooling. The separation was completed when the bromophenol blue reached within 1 cm of the gel bottom. After removal from the apparatus, the gel was stained with 0.5 μg/ml aqueous solution of ethidium bromide for 10 min and then washed in water for an additional 10 min. The gel was then placed in a UV light box and photographed with Polaroid 665 film.

Preparation of Streptavidin-DNA Constructs

Size-exclusion HPLC analysis before and after the addition of an unlabeled construct consisting of the complementary DNA chains bound by their biotin groups to streptavidin was also used to demonstrate that the label was bound to DNA. The extent of the shift in the radioactivity profile to a higher molecular weight after the addition is a measure of radiochemical purity. The streptavidin-DNA constructs were prepared by adding the desired DNA chain at a 1:1 molar ratio relative to streptavidin in saline, pH 6, and incubating for 30 min at room temperature. The constructs were used without purification.

Serum Incubation Studies

After radiolabeling with [99mTc], C-chain was added to fresh human serum at a concentration of 1.2 μg/ml and incubated at 37°C. Samples were periodically removed for size-exclusion HPLC analysis using a single 1.0 × 30-cm Superose-12 column (Pharmacia) and 0.1 M sodium phosphate buffer with 0.15 M saline (PBS) eluant, pH 7.0. The stability of both labels in serum was determined for the radiolabeled C-chain which had been bound, through its biotin moieties, to streptavidin-conjugated magnetic beads. In preparation, suspensions of beads were washed three times with a washing buffer consisting of 10 mM Tris, 1 M NaCl, 0.5 mM EDTA and 0.05% Tween 20, adjusted to pH 7.5. The beads were manipulated for washing by using a magnetic separator. After the third wash, the beads were blocked with a blocking buffer consisting of the washing buffer containing 0.1% calf thymus DNA. The calf thymus DNA averaged 550–750 nucleotides and was stored frozen in a 10-mg/ml aqueous solution. It was diluted 1:10 for use with the washing buffer. The beads were incubated in the blocking buffer for 30 min at room temperature with occasional shaking to block nonspecific DNA binding. The blocking buffer was removed and the beads resuspended in the washing buffer. The suspension was then divided equally. One-half of the sample was blocked with biotin as a control by incubating for 15 min at room temperature with a 100-fold excess of biotin over that necessary for saturation as stated by the manufacturer (i.e., 73 μg biotin per ml of beads at their original concentration). To each half of the sample was then added the labeled conjugated oligonucleotides at a concentration which was about 50% of that necessary for saturation of the streptavidin as stated by the manufacturer (i.e., 19 μg of a 22-base oligonucleotide per ml of beads at their original concentration). The beads were incubated with agitation for 30 min at room temperature and were then washed five times with the washing buffer. The washing buffer was then removed and fresh human serum added to the "dry" beads. The beads were resuspended and placed in a 37°C shaking platform. Aliquots were removed periodically; the beads were separated and
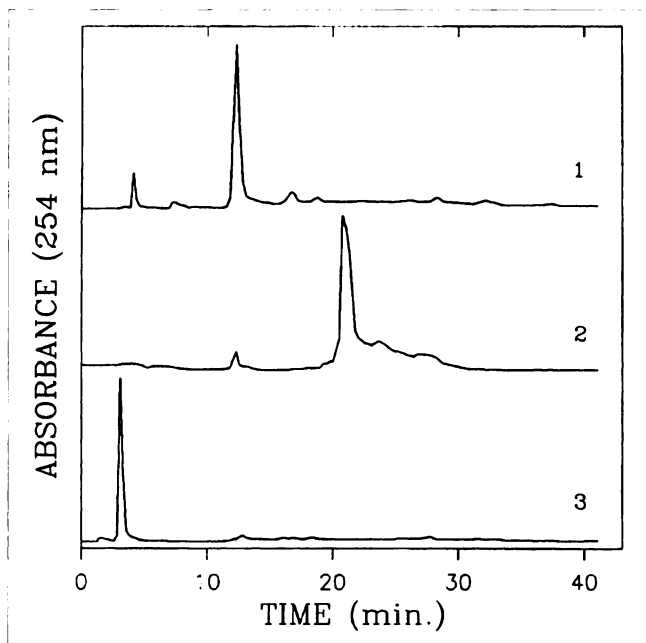
FIGURE 2. UV absorption chromatograms obtained by reversed-phase HPLC analysis of unmodified A-chain (trace 1), of SHNH-conjugated A-chain prior to purification (trace 2) and of DTPA-conjugated A-chain prior to purification (trace 3).

washed three times with the washing buffer. The serum, the combined washes and the beads were then counted in a NaI(Tl) well counter.

Animal Biodistribution Studies

Biodistributions of both ^{99m}Tc- and ^{111}In-labeled D-chain were determined in normal CD-1 male mice. Each animal received by tail vein administration 0.1 ml of saline containing either 7.5 μg (7.3 μCi) ^{111}In- or 10 μg (7 μCi) ^{99m}Tc-labeled DNA. Animals were killed by spinal dislocation 2.5 hr postadministration. Samples of organs were rinsed in cold saline and were counted along with a blood sample and an aliquot of the injectate in a NaI(Tl) well counter. The biodistributions were reported as the percentage of administered radioactivity per gram of tissue.

RESULTS

Oligonucleotide Conjugation

In this study, the amine-derivatized DNAs were conjugated with an NHS derivative of SHNH and with the cyclic anhydride of DTPA. Figure 2 presents UV absorption chromatograms obtained by reversed-phase HPLC of unmodified A-chain (panel 1), SHNH-conjugated A-chain prior to purification (panel 2), and DTPA-conjugated A-chain after purification (panel 3). The unmodified A-chain is clearly resolved into one major peak at a retention time of about 12 min and several minor peaks.

Conjugation with SHNH was accomplished at molar ratios of SHNH:DNA of between 1–25:1. No differences in the ability of the conjugated oligonucleotide to hybridize or to accept a ^{99m}Tc label were observed at these molar ratios (data not presented). Accordingly, all subsequent conjugations were performed at a 1:1 molar ratio.

After conjugation with either SHNH or DTPA, the presence of unmodified A-chain was reduced to a minor constituent (Fig. 2). The peak absorbance shifted to 21 min in the SHNH case and 3 min in the DTPA case. The unmodified DNA peak is essentially absent, demonstrating that conjugation was largely complete.

Because DTPA-coupled oligonucleotides purified on a P-2 column contained unacceptably high levels of unidentified radiochemical contaminant(s) after radiolabeling; purification was ultimately achieved on an anion exchange column. Figure 3 shows the UV absorbance profiles of fractions off this column. The high acidity (pH 2.7) of the initial eluant apparently results in complete protonation of free DTPA which then elutes from the column early. The DNA, however, still possesses a high negative charge through its phosphate groups and is retained. As the ionic strength is increased, however, the increasing sodium ion concentration apparently neutralizes this charge and permits the DNA to elute. The three major peaks (eluting at 20, 100 and 150 ml in the figure) were analyzed by gel
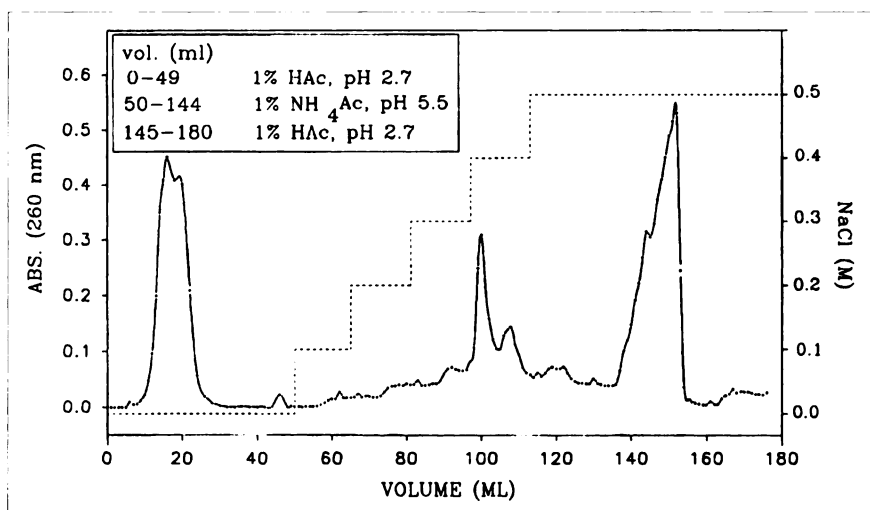

vol. (ml)	
0–49	1% HAc, pH 2.7
50–144	1% NH$_4$Ac, pH 5.5
145–180	1% HAc, pH 2.7

FIGURE 3. UV absorption profile of fractions obtained by anion exchange chromatography of DTPA-conjugated C-chain. Of the three major peaks (at 20, 100 and 150 ml), only the latter was shown to contain DNA.

electrophoresis. Only the latter peak contained DNA (see below). The recovery of DNA in this purification was 16%.

Oligonucleotide Labeling

When 100 μg or more of the SHNH-conjugated DNAs at a concentration of at least 250 μg/ml were radiolabeled with up to 1 mCi ^{99m}Tc, labeling efficiencies ranged between 30%–60%, as determined by Sephadex G-50 chromatography. Attempts to label smaller amounts of DNA or at lower concentrations resulted in reduced efficiencies. These observations were independent of the DNA chain. As controls, the unmodified DNAs were radiolabeled in the identical fashion. Labeling efficiencies were less than 5% in the case of each ^{99m}Tc control. When the ^{99m}Tc-labeled oligonucleotides were analyzed by size-exclusion HPLC, a single peak was always observed. Recoveries occasionally approached 100%, although typical recoveries were 81% ± 5% (s.d., n = 4). That recoveries could be increased by increasing the salinity of the eluant suggests that this retention was due, in part, to ionic interactions of the charged DNAs with the column support.

Typically, specific activities of 50 μCi/μg DNA were achieved when glucoheptonate was replaced with tricine. Properties of the labeled DNAs such as biotin binding, hybridization, protein binding in serum or serum stability appeared to be unchanged with respect to DNA labeled via glucoheptonate.

Labeling efficiencies of the DTPA-conjugated and DEAE-purified oligonucleotides approached 100% under most conditions of DNA concentration and at specific ac-

tivities of 60 μCi/μg. The unconjugated C-chain under identical conditions retained only 2% of the ^{111}In label. Figure 4 presents size-exclusion HPLC radiochromatograms of C-chain radiolabeled with ^{99m}Tc (left column) and ^{111}In (right column) before (trace 1) and after (trace 3) the addition of excess streptavidin. Both labeled DNAs elute in a single peak which largely shifts to a higher molecular weight (i.e., smaller elution volumes) with the addition of the streptavidin. Similar shifts to high molecular weight were observed with the addition of the streptavidin-D-chain (complementary) construct (trace 4). Also in Figure 4 are radiochromatograms showing no shift with the addition of biotin-saturated streptavidin (trace 2). Recoveries in these HPLC analyses were always 80%–90%. Because [^{99m}Tc]pertechnetate is retained, the good recoveries demonstrate that oxidation of the label to pertechnetate was not an important mode of instability.

Electrophoresis Studies

The first four lanes in the electrophoretogram of Figure 5 contain unmodified DNA chains A through D, respectively, stained with ethidium bromide. Whereas chains C and D show only one band at the position expected for a 22-base DNA, that of chains A and B show two distinct bands, one co-migrating with chains C and D plus an additional band with an apparently lower molecular weight. Several other minor bands corresponding to higher molecular weight have occasionally been seen as well. Lanes 5–7 contain aliquots of three peak fractions (at 20, 100 and 150 ml, respectively) from the DEAE anion exchange purifica-
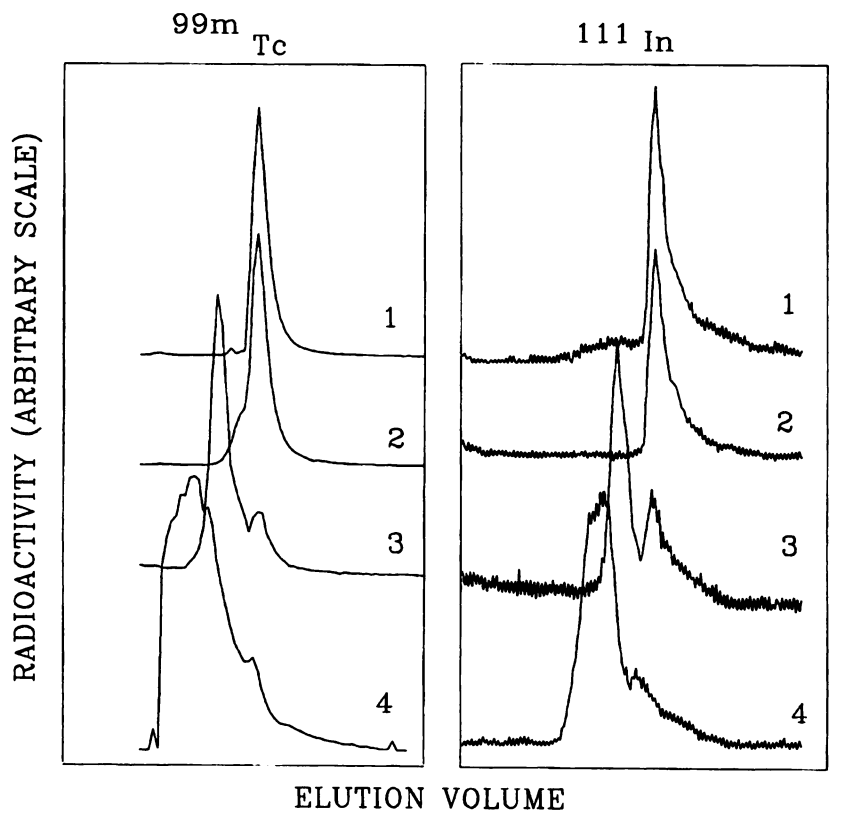

FIGURE 4. Radiochromatograms obtained by size-exclusion HPLC analysis of ^{99m}Tc-labeled C-chain (left column) and ^{111}In-labeled C-chain (right column) in saline (trace 1), in saline containing biotin-saturated streptavidin (trace 2), in saline containing streptavidin (trace 3) and in saline containing complementary D-chain bound to streptavidin (trace 4).
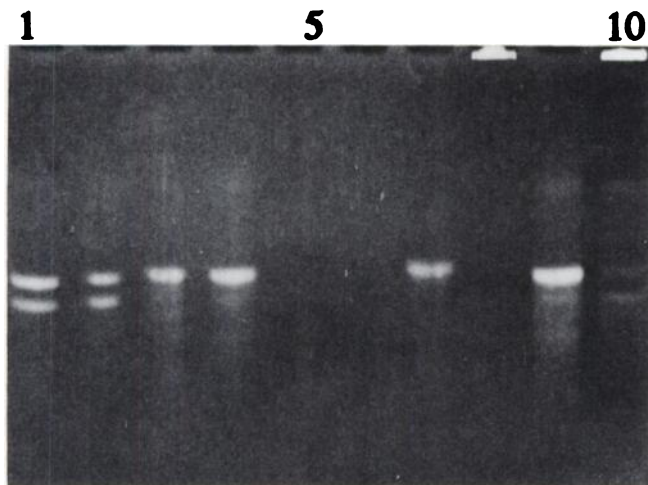
FIGURE 5. PAGE electrophoretogram shows DNA bands stained with ethidium bromide. Lanes 1–4 are unmodified A-, B-, C- and D-chains, respectively, and show the presence of DNA impurities in the A- and B-chain preparations. Lanes 5–7 are aliquots of fractions at 20, 100 and 150 ml, respectively, of an anion-exchange column in the purification of DTPA-coupled D-chain. Only the latter fraction contains DNA. Lanes 7 and 8 are D-chain conjugated with DTPA without (lane 7) and with (lane 8) the addition of streptavidin. Lanes 9 and 10 are D-chain conjugated with DTPA without (lane 9) and with (lane 10) the addition of streptavidin.

tion of DTPA-coupled C-chain (Fig. 3). Only in lane 7 is DNA evident. Accordingly, only the peak eluting at 150 ml off the DEAE column contains DTPA-coupled DNA. That this oligonucleotide has retained its biotin moiety is evident from lane 8 which contained an identical aliquot of the third DEAE peak but mixed with excess streptavidin. All the DNA has shifted to the top of the lane and is therefore associated with a high molecular weight species. Similarly, lanes 9 and 10 are of SHNH-conjugated C-chain with and without the addition of streptavidin, respectively. As before, a complete shift to higher molecular weight is evident after the addition of the protein to the biotinylated DNA.

Serum Incubation Studies

Figure 6 presents size-exclusion HPLC radiochromatograms of serum incubates of C-chain labeled with ^{99m}Tc (left column) and ^{111}In (right column) that show the radiochromatographic profiles before (top panels) and after 1 hr (middle panels) and 24 hr incubations (bottom panels) in 37°C fresh human serum. A rapid shift to a higher molecular weight (i.e., smaller elution volumes) is evident only with ^{99m}Tc and almost certainly signifies serum protein binding. This binding was seen with each oligonucleotide sequence tested. Several studies were performed to establish whether the label had dissociated from DNA prior to binding to serum protein(s). The serum samples were made 25 mg/ml in calf thymus DNA before the addition of labeled DNA. Repeat size-exclusion HPLC measurements after a 1-hr incubation showed an increase of about 40% of the radioactivity co-eluting with the labeled oligonucleotides. In additional experiments, an excess of streptavidin-conjugated beads was added to the serum and, after a 2 hr
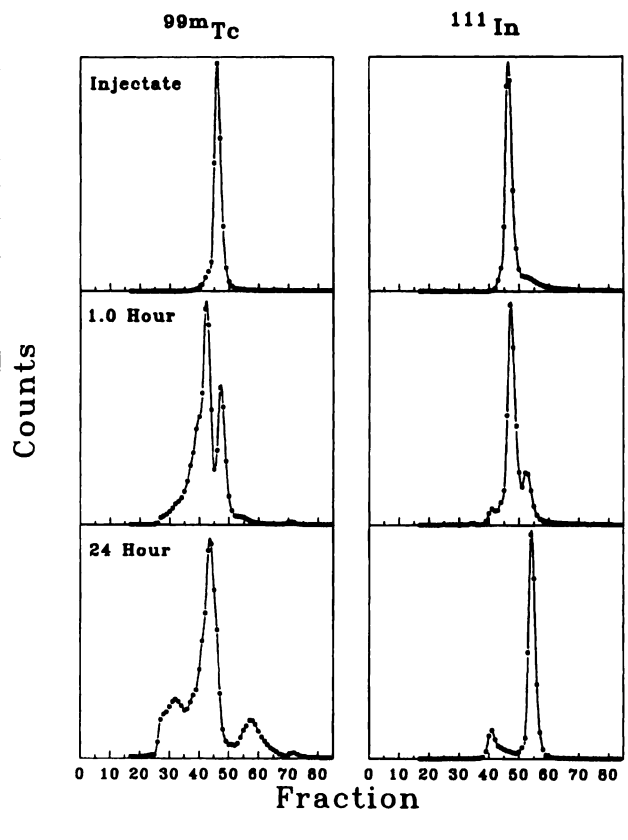

FIGURE 6. Size-exclusion HPLC radiochromatograms of serum incubates of C-chain labeled with ^{99m}Tc (left column) and ^{111}In (right column) show the radiochromatographic profiles before (top panels) and after 1 hr (middle panels) and 24 hr incubation (bottom panels) in 37°C fresh human serum.

incubation with agitation, the beads were washed and counted. About 40% of the ^{99m}Tc was bound to the beads. Although the binding was not complete, the 40% must be compared with a value of about 35%–60% of ^{99m}Tc-DNA which binds to beads under identical circumstances when incubated in saline. The 40% binding from serum therefore shows that the ^{99m}Tc label was bound, at least in part, to one or more serum proteins as the labeled DNA.

Because the serum protein binding of free DNA complicates the determination of serum stability, the oligonucleotides were incubated in serum while bound to streptavidin beads. When presented to the streptavidin-conjugated magnetic beads as described above, 62% ± 1% (s.d., n = 5) of ^{99m}Tc and 51% ± 1% (s.d., n = 5) of ^{111}In was bound. Nonspecific binding, measured with biotin-saturated beads, was 0.7% ± 0.4% (s.d., n = 5) for both labels. Figure 7 presents the percentage of ^{99m}Tc and ^{111}In bound to streptavidin-conjugated beads which was released during serum incubation at 37°C. The figure is based on a single preparation of both ^{99m}Tc- and ^{111}In-labeled C-chain with each preparation added to five serum samples. Only 3.7% ± 3.7% (s.d., n = 5) of ^{99m}Tc and 13.5% ± 4.8% (s.d., n = 5) of ^{111}In dissociated from the beads in 24 hr of incubation.
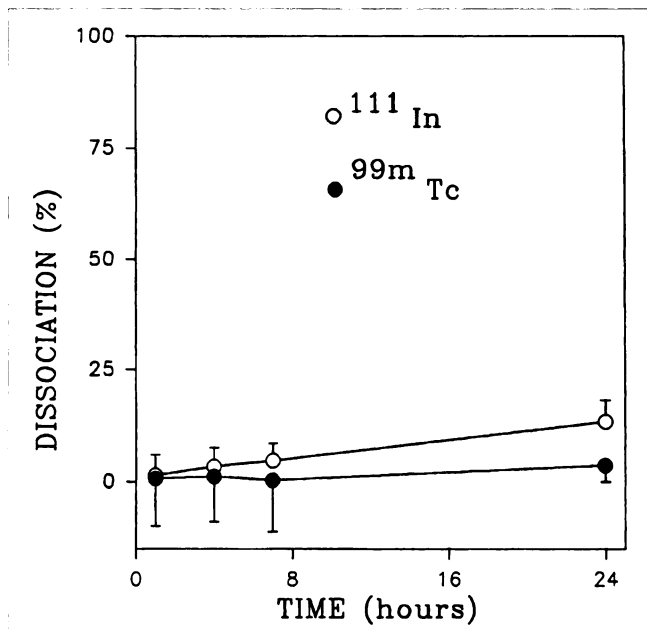
FIGURE 7. Percentage dissociation of ^{99m}Tc and ^{111}In from labeled D-chain bound to magnetic beads during 37°C incubation in fresh human serum for up to 24 hr. Error bars represent 1 s.d. of the mean (n = 5 at all data points).

For the ^{111}In-labeled C-chain, although binding to serum proteins was not observed (Fig. 6), a second peak corresponding to lower molecular weights was seen after 1 hr in serum and which became the prominent peak at 24 hr. Because ^{111}In labeled to proteins by DTPA has been shown to be stable during serum incubations (19), the position of this peak strongly suggests degradation, possibly of the DNA phosphodiester-backbone by nucleases.

Animal Biodistribution Studies

The biodistribution at 2.5 hr postadministration to normal mice are presented in Table 1 as the percentage of injected ^{99m}Tc and ^{111}In. Important differences between labels are readily apparent. The levels of ^{99m}Tc in all tissues were significantly higher than ^{111}In (p < 0.001, Student's unpaired t-test). A partial explanation for these differences most probably is related to the differences in serum protein binding for the two injectates; as demonstrated above (Fig. 6), only the ^{99m}Tc-DNA binds to serum proteins. This would raise activity levels in blood and, by virtue of the blood content of tissues, raise activity levels in tissues as well. From the percentage of organ weight due to blood (20) and from the known blood activity, it is possible to estimate that less than 15% of the activity levels in tissue of Table 1 (other than heart and spleen) were due to the blood pool. The differences between labels may therefore be a consequence of other factors such as instability of the ^{111}In-DNA to degradation in serum.

DISCUSSION

The goal of this study was to develop a method of labeling single-stranded DNAs with ^{99m}Tc which would be

TABLE 1
Biodistribution Results in Normal Mice 2.5 Hours Postintravenous Administration of Technetium-99m and Indium-111-Labeled C-chains

Organ	^{111}In % ID/g	s.d.	^{99m}Tc % ID/g	s.d.
Liver	0.39	0.95	2.5	0.58
Heart	0.03	0.01	0.54	0.12
Kidneys	1.3	0.21	5.6	1.4
Lung	0.08	0.03	0.87	0.23
Stomach	0.21	0.39	2.5	0.67
Spleen	0.1	0.03	0.97	0.27
Muscle	0.04	0.02	0.24	0.08
Skin	0.05	0.01	0.63	0.17
Intestines	0.1	0.11	1.4	1.7
Blood	0.08	0.01	1.8	0.37
	(n = 6)		(n = 6)	

Results presented as percent injected dose per gram (% ID/g) with 1 s.d. of the mean. The levels of ^{99m}Tc in all tissues are significantly higher than ^{111}In (p < 0.001, Student's unpaired t-test).

useful for in vivo imaging applications. Recently, a method of radiolabeling oligonucleotides has been reported in which a derivative of DTPA is covalently attached to an amine group on a modified single-stranded DNA (2,21). The derivitization of an amine-containing DNA with DTPA and with another polyaminopolycarboxylate (ethylenediaminetetraacetic acid) was earlier reported and used for binding stable iron ions (11). DTPA and its derivatives have been useful for labeling proteins such as antibodies with ^{111}In and several other radionuclides (19), but they have been less successful for labeling with ^{99m}Tc because of poor label stability (9). Since label stability was a major concern of this investigation, the SHNH moiety was used as an alternative to DTPA for labeling DNA with ^{99m}Tc. The SHNH moiety was developed for labeling antibodies with ^{99m}Tc (10); this laboratory has demonstrated that, when bound to antibodies by the SHNH moiety, ^{99m}Tc displays acceptable stability in vitro and in vivo (16). Accordingly, the SHNH moiety was considered herein for DNA labeling.

As shown in Figure 2, the primary amino groups attached to the oligonucleotides of this study were readily conjugated with the NHS derivative of SHNH. Following conjugation, the UV peak of the unmodified DNA virtually disappeared on reversed-phase HPLC analysis of the conjugated oligonucleotide. In its place appeared another peak at greater retention time. The results with DTPA are identical except that, in this case, the conjugated DNA eluted earlier than the unmodified DNA. Whereas in past studies from this laboratory of antibody conjugation with SHNH, the presence of high molecular weight protein aggregates was a concern (16), no evidence of DNA aggregation was observed in this investigation. The concern in the use of the cyclic anhydride of DTPA is not aggregation but cross-linking through the two anhydride groups (19). As shown in Figure 5, however, DNA dimers or oligomers, which would

be readily apparent in this analysis, are not evident. Finally, there is little likelihood that treatment with either NHS or cyclic anhydride would derivatize functional groups of the bases themselves (11,22).

The SHNH-DNAs were radiolabeled with ^{99m}Tc following an approach essentially identical to that used routinely for the labeling of SHNH-conjugated antibodies with this radionuclide (10,16). No attempt was made to maximize labeling efficiency, yet typical values were 40%–60% with ^{99m}Tc. Results improved with the substitution of tricine for glucoheptonate as transchelator (17). Labeling at room temperature proceeded more rapidly and the specific activities achievable appeared to be much higher.

In this study, the determination of labeling efficiency and label stability was aided by the use of oligonucleotides derivatized with biotin. The peak ^{99m}Tc and ^{111}In activity in the radiochromatographic profiles obtained by size-exclusion HPLC (Fig. 4) and the migration pattern on gel electrophoresis (Fig. 5) both showed a distinct shift toward a higher molecular weight in the presence of streptavidin. The fact that a similar shift was not evident when biotin-saturated streptavidin was added conclusively demonstrated that the label was on the oligonucleotide. The comparable shift observed when the biotin-saturated, streptavidin-bound complementary chain was added is further evidence. As shown in Figure 7, the biotin moiety was also useful in demonstrating serum stability with the streptavidin-conjugated beads.

That the shifts discussed above were not complete in any case is probably an indication that the unpurified oligonucleotide preparations contained nonbiotinylated DNA chains. For instance, the oligonucleotides used in this study may have been contaminated with a variety of DNA species with different chain lengths or missing the biotin moiety. Polyacrylamide electrophoresis studies (Fig. 5) have clearly shown the presence of lower molecular weight contaminating DNA in preparations of A- and B-chains.

The stability of a label in serum is among the most important factors for an agent under consideration for in vivo use. Because of the high stability of ^{99m}Tc on antibodies facilitated by the SHNH moiety (16), it may not be surprising that ^{99m}Tc showed only 4% dissociation from DNA when incubated on beads in serum (Fig. 7). More meaningful, perhaps, is the stability in serum of the label when incubated in solution. The rapid serum protein binding of ^{99m}Tc-DNAs observed in this study, however, interfered with the determination of stability in that fashion. Nevertheless, some measure of serum stability is evident in that the ^{99m}Tc label was bound, at least in part, to serum proteins by DNA rather than in some "free" chemical form of ^{99m}Tc. This is clear from the large percentage of label which could be prevented from binding to proteins by the addition of calf thymus DNA and which could be displaced by streptavidin-conjugated beads.

It is instructive to speculate on the mechanism of ^{99m}Tc-DNA binding to serum proteins. It is tempting to suggest that the binding may be related to the lipophilicity of the

6-member methylene linker by which the amine is conjugated to DNA. An identical DNA chain but with a hydroxyl-modified 3-carbon linker, however, was also used in this research. Even though this linker is considerably less lipophilic, the serum binding properties of the ^{99m}Tc-labeled oligonucleotide remained unchanged (data not presented). The possibility also exists that the binding may be related to the base sequences selected for this investigation. The same DNA chain, however, showed no tendency towards serum protein binding when labeled with ^{111}In (Fig. 4). Finally, an alternative explanation exists because the SHNH moiety does not, in itself, satisfy the chelation requirements of reduced ^{99m}Tc. Since the complex must be "capped" with glucoheptonate or other species (10), it is possible that serum proteins may participate in this process with the result that the label would be seen to bind to serum proteins. The nature of the binding will need to be established.

Although serum protein binding was not observed for the ^{111}In-labeled C-chain, evidence of degradation upon serum incubation was observed only in this case (Fig. 6). Degradation by nucleases of oligonucleotides with unprotected phosphodiester backbones, such as that used in this research, is a common observation (1). That the ^{99m}Tc-labeled C-chain shows no evidence of labeled degradation products during serum incubation may be a consequence of the serum protein binding.

CONCLUSION

Oligonucleotides derivatized with a primary amine can be conjugated with SHNH and radiolabeled stably with ^{99m}Tc. The stability of the label is comparable to that observed by us for ^{111}In radiolabeled to the same oligonucleotides by DTPA. One interesting observation from this work is that ^{99m}Tc-DNA, when labeled in this fashion, binds to serum proteins. The binding is probably related to the SHNH moieties since similar binding was not observed for ^{111}In-DNA. A consequence of this binding was higher ^{99m}Tc blood levels in vivo, but another consequence may be increased stability to nucleases in serum since significant degradation products were observed in this work only for ^{111}In. Further investigations should establish whether serum binding of ^{99m}Tc-DNAs will interfere with targeting in vivo.

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