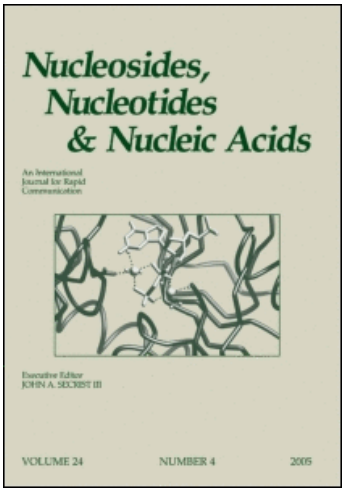

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High Specific Radioactivity Labeling of Oligonucleotides with ^{3}H-Succinimidyl Propionate

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HIGH SPECIFIC RADIOACTIVITY LABELING OF OLIGONUCLEOTIDES WITH ^{3}H-SUCCINIMIDYL PROPIONATE

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ABSTRACT

An easy and rapid method for tritium labeling of deprotected oligonucleotides is proposed. The method consists in performing the reaction of commercial ^{3}H-succinimidyl propionate with a terminal amino group of the oligonucleotide in an organic medium. High specific radioactivity labeling can be achieved with minimal radiolysis during long term storage. The synthesis of the nonradioactive congener having an identical structure to the labeled compound is also described.

More widespread and useful applications of oligodeoxynucleotides (ODN) in antisense technology could be realized if chemists were able to propose convenient and easy methods for obtaining oligonucleotides resistant to nuclease attack and easily labeled for *in vivo* studies. Although several labeling methods have been set up in the past, very few have appeared as fully satisfactory for convenient, safe or long term utilization. Radiolabels such as ^{32}P or ^{35}S containing phosphate groups were among the first chemical moieties to be used for the labeling of oligodeoxynucleotides. However, easy removal of the radiolabel by phosphatases, short half-lives and the necessity to protect workers against beta radiations were the main drawbacks in the utilization of these isotopes. To prevent the loss of the radioactive phosphate group, the radiolabel was introduced at the internucleotidic linkage as internal labeling. Sulfurization by $^{35}S_8$ was thus performed on support-bound H-phosphonate oligomers (1). Similarly, Levis et al.

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(2) prepared tritium-labeled oligomers on the phosphonate linkage for cellular uptake measurements. In studies of transport of oligonucleotides in the intestines of rat, ^{14}C-labeled-oligomers were synthesized by reductive alkylation with ^{14}C-formaldehyde (3). Nucleobases labeling has been carried out by exchange reactions at the C-8 of purines with tritiated water (4). Other work has dealt with the preparation of ^{14}C- or ^{3}H-dimethoxytrityl-nucleosides for incorporation during machine synthesis (5). Other types of labeling involving the ^{125}I isotope included the end-labeling of amino-containing oligonucleotides with the ^{125}I-Bolton Hunter reagent for RNA detection (6) or the synthesis of ^{125}I-cytidine, which once incorporated within the chain of the antisense oligonucleotide, can induce strand scission of the complementary target chain (7,8).

In contrast to most of the methods cited above which involved reactions on fully protected oligonucleotides during machine synthesis, we describe in this paper a ^{3}H-labeling method that can be easily performed with a commercial tritiated reagent on deprotected oligonucleotides. The only requirement is the presence of a primary alkyl-amine function in the oligomer structure. The reaction is based on the classical nucleophile attack of amino groups on succinimidylated carboxyl groups of tritium-labeled carboxylic acids. To prevent nuclease attack that occurs most often at the 3' side, the reacting amino group is advantageously tethered on this side of the oligomer molecule. High reaction yields could be achieved by performing the reaction in a water-free organic medium, and this was possible by complexing the oligomer molecule with a lipocation. Our results showed that high radioactive labeling (>25 Millions cpm/nmol) of oligomer samples can be obtained by this method within a day including the purification procedure. Although the labeled compounds were shown to remain fairly stable, loss of the label from the oligonucleotide was found to be limited (<10%) after 3 months of storage at -20°C. The proposed method could find useful applications in antisense technology for not only tracking the antisense molecule in cultured cells but for organs distribution studies as well.

MATERIALS AND METHODS

Oligonucleotides, Chemicals

Oligodeoxynucleotides were synthesized by Eurogentec (Seraing, Belgium). Amino groups were tethered to the terminal phosphate (p) with various linkers "amino links" from Glenn Research:

C3 = 3'-p-CH_2-CH_2-CH_2-NH_2

C6 = 5'-p-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-NH_2

C7 = 3'-p-CH_2-CH_2-CH_2-CH_2-CH-(CH_2OH)-CH_2-NH_2

One compound (oligomer 17 in Table 2) had the amino group attached at the C-5 position of the terminal thymine base on the 5' side via the following linker: -CH_2=CH-CO-NH-$(CH_2)_6$-NH_2 (the amino-modifier C6 dT of Glenn Research), while the 3' end was derivatized with a C7-Fluorescein moiety.

Purification consisted in treating oligomers by butanol extraction followed by ethanol precipitation. Sequence, length and end modifications are presented in Tables 1 and 2. Purity and integrity of labeled ODN were assessed by ion-exchange HPLC equipped with a radioactivity detector (Berthold 506C-1). The column (PE TSK DEAE-NPR column from Perkin-Elmer) was a weak anion exchange column containing diethylaminoethyl groups bonded to hydrophilic polymer beads. Buffer A contained 20% acetonitrile in potassium phosphate buffer 10 mM, pH 7 and Buffer B, 20% acetonitrile in potassium phosphate buffer 10 mM, pH 7 and KCl 1 M. The elution gradient conditions were 0-5 min: 0% B, 5-25 min: 0-60% B. The separation of the 3'-propionyl from the 3'-amino oligomer was performed by reversed phase HPLC on a C18 column (150 x 4.6 mm) under elution gradient conditions as follows: 0-50 min: 0-18% buffer B; 50-55 min: 18-0% buffer B. Buffer A contained 5% acetonitrile and 95% triethylammonium acetate 0.1 M, pH 7 and buffer B contained 80% acetonitrile and 20% triethylammonium acetate 0.1 M, pH 7.

The radioactive reagent, N-Succinimidyl (2,3-^{3}H) propionate was from Amersham France S.A. The sample used throughout this work had a specific activity of 100 Ci/mmol at a concentration of 1 mCi/ml in toluene solution. Cetyltrimethylammonium bromide (CTAB), tris(hydroxymethyl)amino-methane (Tris), ethylenediaminetetraacetic acid (EDTA) and propionic anhydride were purchased from Sigma. Dimethylsulfoxide (DMSO), methanol, toluene were of reagent grade.

Ultraviolet-visible spectra were recorded on a Beckman DU 640 spectrophotometer. Scintillation counting was carried out on a LKB Wallac 1212 Rackbeta counter.

Formation of oligonucleotide-CTAB complexes

In a standard reaction, 1-5 nmol of ODN were dissolved in 30 µL of water. The CTAB cation was added from a concentrated aqueous solution (25 nmol/µL) at a ratio of 1 to 2 CTAB per phosphate group. A cloudy precipitate appeared and the precipitation could be completed by 3 rounds of freeze-thaw in liquid nitrogen. The precipitate was then isolated by centrifugation (12000 g, 30 min at 4°C). The supernatant was removed

and the quantity of the nonprecipitated oligomer could be measured by absorption spectroscopy. Usually the yield of the oligomer precipitation by CTAB was higher than 95%. The precipitate was then dried under vacuum, redissolved in 50 μL methanol, evaporated and redissolved in 50 μL of toluene-methanol (3:2 by volume) and evaporated again to remove traces of water. Finally the dry precipitate was dissolved in 20-50 μL of dry DMSO and stored as a stock solution of the ODN-CTAB complex.

Labeling reaction

To a defined quantity of the ODN-CTAB complex in DMSO solution in a microcentrifuge tube was added the radioactive reagent in toluene solution. The stoichiometry of the two reactants determined the specific radioactivity of the labeled sample. The volume of toluene should not exceed that of DMSO as the reaction yield drops when the volume of toluene is too high. The toluene can be removed under vacuum but concentrating the reaction medium to dryness is not recommended because of the volatility of the radioactive material. At the end of the reaction, the reacted and the non-reacted oligonucleotides were recovered as a precipitate by displacing the CTAB cation by 10 fold sodium dodecylsulfate (SDS) and by adding NaCl or sodium acetate (0.3M) and ethanol. The precipitation was carried out at -20°C for 2 h or could be shortened to 1 min by dipping the tube in liquid nitrogen. The oligomer precipitate was assembled by centrifugation (12000g for 30 min at 4°C), redissolved by 100 μL of water, reprecipitated as above and washed by cold ethanol. The pellet containing the labeled oligonucleotide was finally redissolved in 100 μL of water. The specific radioactivity of the labeled sample was determined by measuring the absorbance and the associated radioactivity of an aliquot of the labeled oligomer solution. Aliquots of ethanol washes were also counted in order to follow the purification procedure and for calculating the total radioactivity of the reaction medium. The yield of labeling was expressed by the ratio of the radioactivity of the pellet to the total radioactivity of the reaction medium.

Synthesis of the nonradioactive congener

Antisense studies in cultured cells or in model animals most of the time, involved dose response experiments. This implies that unlabeled ODN is utilized with the labeled oligomer for adjusting concentrations. It is also desirable that the added oligomer have the same chemical structure as the labeled oligomer. The protocol of radioactive labeling as presented above should be applicable to the synthesis of the nonradioactive congener. It turned out that the corresponding N-succinimidyl propionate was not commercially available. We then looked for an alternative way for tethering a propionyl group to the 3'-amino-ODN. The amidation reaction of the terminal amine group with a propionyl group

could be performed by reaction of propionic anhydride on the 3'-amino-oligonucleotide. To a concentrated solution of propionic anhydride in DMSO (50 µL) was added an equal volume of ODN in aqueous carbonate buffer (50 mM sodium carbonate, pH 9) and left at 4°C for 16 h. A large excess of the anhydride over the primary amino group (x 2000) was used to compensate for the strong hydrolysis reaction of the anhydride with water and for complete amidation of the amino groups. A slight reaction on the exocyclic amino groups of nucleobases could occur under these conditions. To regenerate the nucleic bases, alkaline treatment of the modified oligomer in 1 M aqueous ammonia solution was performed overnight. The modified oligomer was recovered and purified by ethanol precipitation. The chemical modification and deprotection reaction were followed by reversed phase HPLC.

RESULTS AND DISCUSSION

When the labeling reaction was performed in an aqueous medium in standard conditions as specified in Table 1, the yield of label transfer was found to be very low (less than 2%), very likely due to the strong hydrolysis reaction at the expense of the labeling reaction. To avoid the loss of the radioactive reagent by hydrolysis, it would obviously be more advantageous to perform the reaction in an organic medium. Complexes of oligonucleotides with the lipocation CTAB were found to dissolve in various organic solvents such as methanol, dimethylsulfoxide, mixtures of toluene-methanol, pyridine etc... In the past, we and others (9-11) have used this method for chemical derivatization of unprotected oligonucleotides. The same method was used to precipitate DNA from cell extracts either with CTAB (12) or with other hydrophobic cations (13). In this study we used this method to dissolve the unprotected oligonucleotide in an organic solvent such as DMSO for reaction with the ^{3}H-labeled succinimidyl propionate dissolved in toluene. High yields of the reaction on the alkyl-amine groups were achieved in an organic medium while side reactions on exocyclic amines were negligible as shown below.

Comparative reactivity of terminal primary amino and exocyclic amino groups of oligonucleotides

Labeling assays were performed on two series of oligomers which were classified according to the chemical nature of their 3' termini. Reaction conditions are given in the caption of Table 1.

TABLE 1

Yields of label transfer to oligonucleotides with different backbones or 3' end structures.

N°	Backbone	Length	Sequence	Yield[a](%)
			Class I: 3'-OH	
1	PO	15mer	AAC GTT GAG GGG CAT	< 2
2	PO	16mer	TTT TTT TTT TTT TTT T	< 2
3	PO	18mer	GAA CGG CAT TTC ATC TCT	< 2
4	PS	20mer	TCC GGA GCC AGA CTT CAT TC	< 2
5	PS	25mer	CTC TCG CAC CCA TCT CTC TCC TTC T	< 2
			Class II: 3'-C7-NH_2	
6	PO	18mer	CGG CAC AGC ATA TAT AG	80
7	PS	20mer	TCC GGA GCC AGA CTT CAT TC	80
8	PO	20 mer	AGC GGT CCC ACT CTT GTT TG	88
9	PO	20 mer	GAG TCC AGA GCC TCC TTA CT	90

[a]Reaction conditions: ODN (2 nmol), ^{3}H-succinimidyl-propionate (0.1 nmol) in 30 µL DMSO-toluene solution, 3h at room temperature. The yield is expressed as the percentage of the radioactive label transferred to the oligonucleotide molecule.

Compounds in class I represent oligomers that are terminated by a 3'-OH group while those of class II are tethered to a primary amino group at their 3' ends. In class I, compound 2 is a hexadecathymidilate that does not possess any exocyclic amino group in its structure while all the other compounds contain exocyclic amino groups. It is interesting to note that the yields of labeling of class I compounds were very low (usually 0.5-2%) suggesting that reactions on exocyclic amines, if any, were negligible under our experimental conditions. In contrast to this, labeling yields for class II compounds were

very high (>80%) showing an efficient coupling reaction of the terminal primary amine with the activated carboxyl group of the radioactive reagent. The difference in the reactivity of the terminal primary amine and the exocyclic amines could be illustrated by the difference in the yields of labeling (values in parentheses) of the two oligomers 4 (1%) and 7 (80%) which bear the same sequence and are made of the same backbone (PS). The results presented in Table 1 also show that the yield of labeling did not depend on either the length, sequence or nature of the backbone (PS or PO) of the oligonucleotide molecule.

Oligonucleotides and especially those made of a phosphodiester backbone will exhibit prolonged lifetime *in vivo* provided their 3' ends are suitably protected (14) as 3'-exonucleases have proven to be the most active in biological media (15). Enhanced stability of 3'-amino containing oligonucleotides was observed in cell culture (14,16) and therefore the present labeling method is particularly suitable for radioactive labeling of these amino-modified compounds.

Effect of temperature on the reaction kinetics

The kinetics of the labeling reaction of oligomer 7 at three different temperatures are presented in Figure 1. The kinetic curves reached a common plateau value of ca. 85% of the transfer of the label to the oligonucleotide molecule. This corresponds typically to specific activity values of 20-25 million cpm/nmol ODN. At room temperature it took more than 3 h to reach the plateau value while at 60°C it took only 15 min to reach almost the same yield of label transfer. Increasing the temperature resulted in the shortening of the time needed to reach the plateau value. Morever the temperature did not seem to favor side reactions on exocyclic amine groups as the yields of labeling after a 30 minute reaction of compound 4 at 60°C (<1%) was as low as that observed at 20°C (<2%) for the same reaction duration.

Influence of the chemical structure of oligomers on the yield of labeling

In Table 2 we compared the labeling yield of a series of oligomers in which various chemical modifications were introduced.

All the oligomers presented in Table 2 bear a terminal amino group at the 3' end except for oligomer 16 in which the amino group is derivatized at the 5' end via a C6 linker and oligomer 17 where the amino group is linked to the oligonucleotide at the C-5 of the 5' terminal thymine. Compounds 7, 10 and 11 have the same sequence but differ only in the type of backbone or the presence of a fluorescein group at the 5' extremity (compound

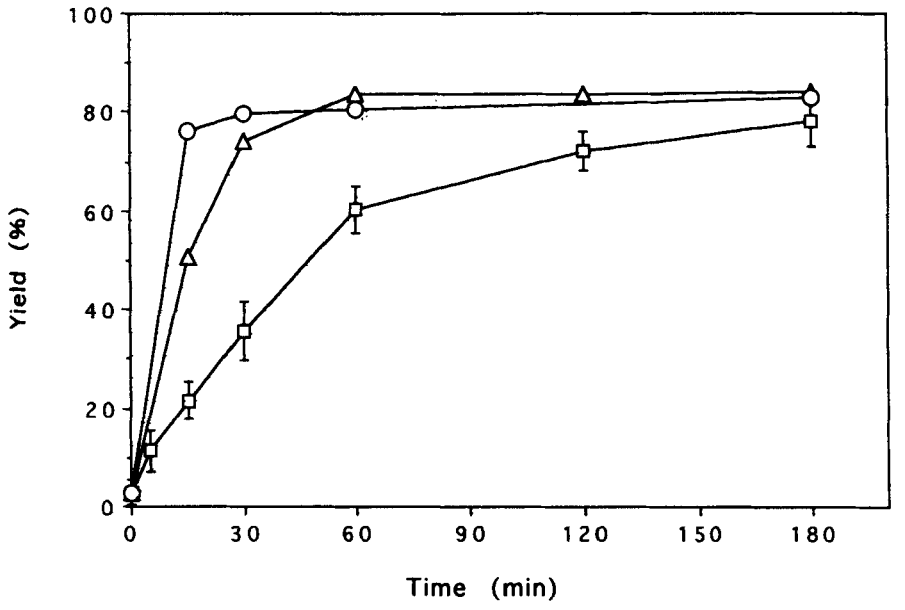

FIGURE 1: Effect of temperature (□ : 20°; Δ : 37°; O : 60°C) on the kinetics of the labeling reaction. The reaction mixture contained: ODN (Oligomer 7 at 130 μM), the radioactive reagent (6.7 μM) in 30 μL DMSO-Toluene solution. Error bars represent standard deviations of 3 assays.

11). The corresponding labeling yields showed that neither of these differences had any influence on the labeling reaction. Absorption spectra and HPLC profiles of oligomer 11 before and after labeling were found to be identical showing that the labeling conditions preserved both the integrity of the fluorophore and the nucleobases. This is particularly interesting for the preparation of double-labeled oligomers for studies such as cell internalization and distribution. Another set of identical sequence compounds such as 12, 13 and 14, once again showed that the replacement of the oxygen atom by a sulfur atom in the backbone did not significantly affect the efficiency of the labeling reaction. However the labeling yield dropped when the structure of the terminal phosphodiester bonds was modified like in compound 14 in which the 2' carbon of the ribose was derivatized with an O-methyl substituent. In this particular case we noticed that the CTAB salt was not fully formed probably leading to altered solubility of the complex in the organic medium. A similar situation in terms of yield drop was observed when the amino group was attached to a short linker (C3) as in compound 15. In either case a peculiar structure may appear at the 3' side of the oligomer that could result in reduced accessibility of the NH_2 reacting group to the labeling reagent. Unsuccessful attempts

TABLE 2

Yields of label transfer in chemically-modified oligomers

N°	Back-bone	Length	Sequence	Yield[b] (%)
7	PS	20mer	TCC GGA GCC AGA CTT CAT TC'	80
10	PO	20mer	TCC GGA GCC AGA CTT CAT TC	91
11	PO	20mer	Fluo-C7-5'-TCC GGA GCC AGA CTT CAT TC	80
12	PS	15mer	CAT GGT CTG CCC TGA	81
13	PO	15mer	CAT GGT CTG CCC TGA	64
14	PO	15mer	C*A*T GGT CTG CCC T*G*A*	9
			(Oligomer 14 contains 2'-O-Methyl internucleotide linkage at * positions)	
15	PS	12mer	GGA CGT GAC TGT-3'-C3-NH_2	1
16	PO	19mer	NH2-C6-5'-GGC TCC ATT TCT TGC TCT C	62
17	PO	20mer	T^{NH2}CC GGA GCC AGA CTT CAT TC-C7-Fluo	61

[b]Same conditions as in Table 1. All compounds carry a 3'-C7-NH_2 terminal group except for compounds 15, 16 and 17.

were made to improve the labeling yield either by adding triethylamine to the reaction medium as a proton capture agent or by heating the oligomer in a hot ammonia solution prior to labeling, to remove any remaining protecting group from the oligomer.

Finally we tested the possibility of performing the reaction on amino groups located at other sites of the oligomer molecule. Using the same method, we succeeded in labeling the amino group attached to the 5' end of an oligonucleotide as exemplified by the labeling of oligomer 16 or an amino group tethered to the C-5 of a pyrimidine such as the thymine base in 17.

Synthesis of the nonradioactive congener

The amidation reaction was performed by adding a large excess (x 2000) of propionic anhydride in DMSO to a prechilled (4°C) aqueous solution of the amino-

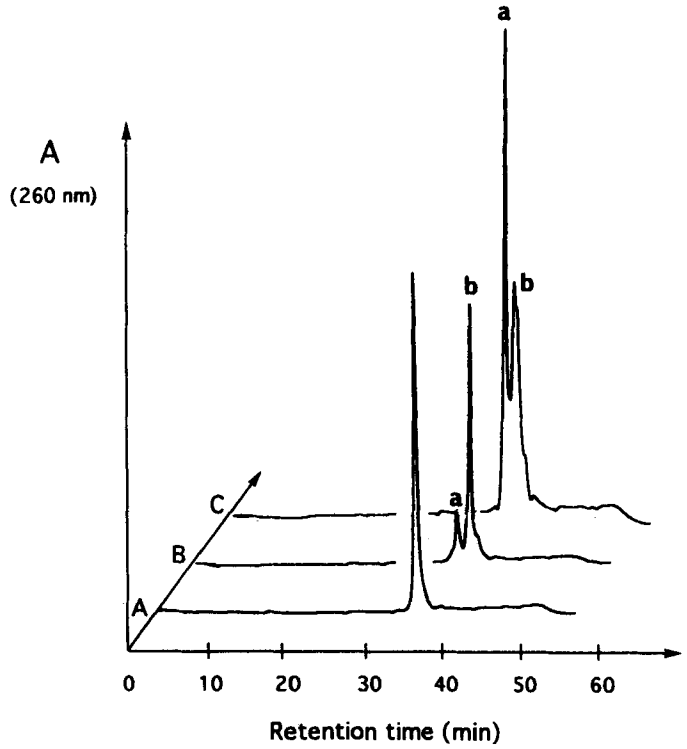

FIGURE 2: Reversed phase HPLC chromatograms showing elution profiles of A: oligomer 10 (elution time = 37.7 min); B: reaction mixture of 10 with propionic anhydride followed by alkaline treatment and precipitation by ethanol (peak a = 37.4 min; peak b = 39.2 min) and C: the reaction mixture as in B in which a small amount of 10 was added (peak a = 37.9 min; peak b = 39.4 min). For details of the amidation reaction and the elution conditions see Materials and Methods.

containing oligomer which was left overnight at 4°C. The anhydride was removed under vacuum and the pellet redissolved in 1 M NH_4OH and left for at least 6 h for cleavage of any of the "propionyl protecting" groups from the exocyclic amines. Alkaline treatment did not cleave the amide bond because in a separate experiment, we were able to recover, by precipitation, the quasi-totality of the radioactivity associated to a labeled oligomer after treatment with the NH_4OH solution. Addition of a propionyl tail to the end of the oligonucleotide could introduce a hydrophobic character to the molecule that can be reflected in the difference in elution properties of the modified oligomers on a reversed phase column. Analysis by reversed phase HPLC of the labeling reaction of oligomer 10 is shown in Figure 2.

Compared to the starting compound (chromatogram A), it can be seen that after reaction with propionic anhydride and alkaline treatment, compound 10 almost disappeared and was converted into a new compound eluting at a longer retention time (chromatogram B, peak b). Chromatogram C corresponds to the injection of the treated product (as in B) but to which we added the original compound 10 in order to confirm its position in the elution profile. Retention times are given in the Figure 2 caption. From these results it can be stated that peak b corresponds very likely to the 3'-propionyl oligonucleotide. Another proof for the amidation reaction of the primary amino group is provided by collecting the compound corresponding to peak b, which was then purified and allowed to react with the radioactive reagent. The yield of labeling was found to be very low (2-3%) confirming that the primary amine groups were totally blocked by the anhydride reagent. The above results suggest that a 3'-propionyl-ODN was formed by reaction of a 3'-amino-ODN with propionic anhydride under the described conditions. This "cold" congener of the radioactive ODN could be useful for studies such as cell penetration in which the effect of different oligonucleotide concentrations may be studied while maintaining the radioactivity constant over the cells. Finally because reversed phase HPLC allowed the labeled oligomer (propionyl form) to be isolated from the amino form (unlabeled oligomer), the preparation of tritium-labeled samples with maximum specific radioactivity (theoretically 222 million dpm/nmol) is therefore feasible.

Stability of the ^{3}H-labeled ODN in the presence of cells

The ^{3}H-labeled PO-ODN 10 was assessed for its stability in the presence of vascular smooth muscle cells (VSMC). Oligomers were added to the culture medium in 35 mm dishes containing ca. $5x10^{5}$ cells and incubated for 2 h at 37°C in a humidified 5% CO_2 incubator. Two ODN concentrations 0.1 and 1 µM were tested in which the radioactive ODN was set at 1 million cpm and the final ODN concentration was adjusted by adding the cold ODN congener. Aliquots of the culture medium was directly injected in a HPLC anion-exchange column. The HPLC profile presented a series of small peaks eluting between the injection signal and a main peak eluting at 23 min corresponding to the original compound. The results showed that after 2 h of incubation in the presence of VSMC more than 85% of the added ODN remained intact. There was a slight effect of the ODN concentration on the stability of oligomers as more than 90% of the added ODN instead of 85% were found intact when used at 1 µM. As expected, PS-ODN were found more stable than PO oligomers in the presence of the same cells (VSMC).

We also measured the retention time of 2,3-^{3}H-propionic acid, the hydrolysis product of the radioactive reagent, N-succinimidyl- 2,3-^{3}H-propionate under the same elution conditions. In any case we found the presence of radioactive propionic acid in the

supernatant of cells after 2 h of incubation. This is an indication that the carboxamido bond that links the label to the oligonucleotide was not disrupted in the presence of VSMC.

Storage

The stability of the labeled ODN as checked by anion exchange HPLC (results not shown) was found quite satisfactory, with a slight loss of the radioactivity (<10%) observed after a 3 month storage period at -20°C. The labeled oligomer was kept in Tris,HCl buffer (10 mM, pH 7.8), EDTA 1mM and ethanol (15%). Addition of thiols like beta-mercaptoethanol as radical scavengers have been shown to reduce radiolysis of ^{3}H-labeled oligonucleotides during storage (4).

In summary the reaction worked up in this study presents some definite advantages over existing methods of radioactive labeling of oligonucleotides. The proposed method is easy to set up and can be used on any commercial deprotected oligonucleotide. High yields of the label transfer reaction made it possible to reach high specific radioactive oligomers which can then be stored for months (or even years) as any other "ready to use" chemicals. We are currently using the ^{3}H-labeled oligonucleotides for uptake and compartmentalization studies in vascular smooth muscle cells.

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