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Review

Qualitative and quantitative measurements of oligonucleotides in gene therapy: Part I in vitro models

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Abstract

Part I of this review attempts to bring together all the methods of detection and determination of synthetic oligonucleotides used in in vitro, described in the literature over the past 14 years, in an effort by scientists to use these oligonucleotides as drugs in gene therapy. The in vitro models include cell-free and cell culture systems. Emphasis has been given to the techniques developed for quantification of the input oligonucleotides or their metabolites. The purpose of study, methods of processing, detection and determination techniques such as those based on fluorescence, radiolabeling, high-performance liquid chromatography, gel-electrophoresis and others have been presented. © 1997 Elsevier Science B.V.

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1. Introduction

A therapeutic response as a result of targeting proteins is the main thrust of the majority of drugs on the market or at developmental stage [1]. The first chemotherapy by alkylating agents for treatment of cancers (i.e. Hodgkin's disease and lymphomas) was done in 1943. The principal problem with conventional chemotherapeutic agents, including alkylating agents, anthracyclins, anti-folates, anti-tumor antibodies and tubule synthesis inhibitors, is that the biochemical resistance generally occurs in all instances in patients with solid tumors [2].

Gene expression in both prokaryotes and eukaryotes is controlled by the products of regulatory genes. Naturally occurring regulatory genes have been discovered that direct the synthesis of RNA and can directly control gene expression. These newly discovered RNA repressors are highly specific inhibitors of gene expression. The regulatory RNA contains a sequence that is complementary to the target RNA and binding of the

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two RNAs occurs by base pairing. The term 'antisense RNA' has been coined to designate this regulatory RNA [3].

The two new fields of therapeutic developments (i.e. antisense and gene therapy) should be considered as a unified field of genetically targeted research and therapeutic development because both involve the design of functional nucleic acids targeted to manipulate physiological processes through genetic intervention. Hence, this review encompasses both fields in the term 'gene therapy' [4].

A review article [5] on gene therapy protocols to suppress malignant tissue growth both in animal models and clinical trials discussed various approaches of gene-transfer to malignant sites using both the antisense approach to suppress oncogenes and retroviral transfer of inhibitory genes. However, how the genes function in vivo and the fate of the transferred gene have not been fully defined. The concept of reduction of specific gene expression by its direct inhibition, or by interfering with its product, is finding strong grounds for both therapeutic and experimental reasons. The exogenous inhibitors of gene expression such as antisense RNA/DNA, which are targeted at specific messenger RNA, are being developed and evaluated in different laboratories with rapid advances in gene therapy [6,7]. The antisense technology is based on the principle of specific interference with either expression or function of genes regulating biological processes. This is achieved by saturating the cell with a nucleotide whose sequence is complementary to a portion of the mRNA encoding the protein (Fig. 1). Chemically synthesized short complementary DNA sequence oligodeoxynucleotide can be introduced into a cell. Alternatively, the cell can be transfected by a plasmid in which the gene encoding the protein is in opposite orientation with respect to the promoter. The resultant duplex is a DNA/RNA hybrid as a substrate for an enzymatic activity, RNaseH, which specifically degrades the RNA strand in the duplex [8–12] (Fig. 1). The latter approach of recognizing cellular sense RNA is widely termed as the antisense DNA approach [10]. The goal is to develop small oligonucleotides, plasmids or retroviral vectors which can be easily introduced into living cells so as to inhibit the specific gene expression either at transcription (antigene) or translation level (antisense DNA). Oligonucleotides are relatively flexible, rodlike molecules, typically 15–30 nucleotides long, having molecular weights between 4500–10 000 kDa. In the literature, ^{14}C-, ^{35}S-, ^{32}P-, ^{3}H- and ^{125}I-labeled oligonucleotides have been used both for in vitro and in vivo studies. For details of labeling oligonucleotides with these radioactive nuclides, readers are directed to refer to the specific citations wherever they appear. Elaborate reviews have discussed the (1) principles of antisense usage, (2) various modifications to enhance cellular uptake, distribution and stability besides other related aspects of the antisense gene therapy [3,7,13–23]. The essential requirements of (1) gene specific therapeutics, (2) backbone modifications, (3) pharmacodynamics, (4) possible mechanisms of actions or (5) pharmacokinetics of this class of drug are outside the scope of this review and interested readers are referred to other articles [24–26].

In order to understand the effect and mechanisms of action of these synthetic oligonucleotides, enormous efforts have been made over the past two decades. The first reported use of a synthetic antisense DNA oligonucleotide was in 1978 [27]. To this goal, research has been conducted to develop both in vitro and in vivo models of uses and effects of the synthetic oligonucleotides. Very few rigorous studies sepa-

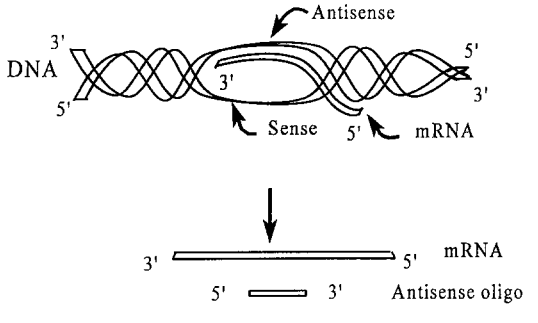

Fig. 1. Propsed mechanism of action of antisense oligonucleotide. The DNA strand unfolds to synthesize mRNA from the antisense strand of the DNA. An antisense oligonucleotide intended to inhibit synthesis of a protein from the mRNA, covalently binds with a portion of the mRNA.

rate intact oligonucleotides from degradation products and address the kinetics of drug disposition [26]. Some researchers simply studied the effect of oligonucleotides on cell cultures, while some studied the stability of these in cell-free systems. Detection and quantification of the input oligonucleotides in cells, animals and human trails as part of pharmacokinetic analysis have been tried by many investigators. Since the literature is replete with enormous variations in the methods used to qualify or quantify these oligonucleotides, it seemed reasonable to compile all these analysis techniques in a review for a vast scientific community involved directly or indirectly in therapeutic/clinical uses of this new generation of oligonucleotides. Hence, this review, Part I, presents the methods used to determine the input oligonucleotide and covers most of the work done in cell-free and cell-culture. Part II of this review deals with those methods for determination of oligonucleotides in non-primate and primate models.

In vitro testing as a method of selecting chemotherapeutic agents has been employed for developing specific drugs. These are either clonogenic assays or short term culture in defined media or, occasionally, chemical tests. On the whole, these tests have not been applicable to the successful treatment of human cancers [28]. Different systems and techniques for measurement of input oligonucleotides are discussed below.

2. Cell free systems

To overcome the complexity of investigation in intact cells, several laboratories have developed cell-free models to evaluate the inhibitory potential of these molecules. Nevertheless, cell-free studies have usually been limited to observations at the translational level and are not always representative of the intact cell situation [7,9]. In cell free systems, comparative stability and inhibitory activities have been studied using both fluorescence and radioactive labeled oligonucleotides.

2.1. Fluorescence

Thenet et al. [29] have studied the biological stability of synthetic oligonucleotides with α- and β-anomeric configuration at the carbon 1′ of the deoxyribose sugar in NIH 3T3 cellular extracts. The oligonucleotides were (a) α- and β-tetrathymidylates covalently linked to an intercalating agent oxazolpyridocarbazole through a pentamethylenic arm (T4-C5-OPC) and (b) α- and β-hexa-adenylate (A6). NIH 3T3 cells at a density of 6000 cells μl^{-1} in buffer were sonicated under mild conditions and were incubated with the oligonucleotides for different incubation times. The sample aliquots were analyzed by thin-layer chromatography on silica gel (DC-Alufolien Kieselgel 60F 254, Merck). The mobile phase was isopropanol–ammonia–water (4:1:1, v/v/v). The oligonucleotides and their degradation products were detected by UV (366 nm for oligonucleotides bound to an intercalating agent and 254 nm for non-bound ones) and quantified by densitometry. They concluded that (i) α-structure confers an improved stability in cellular extracts as compared to β-structure, and (ii) the intercalation at the 3′ position has a synergistic effect to α-anomeric oligonucleotide on the stability [29].

The nuclease susceptibilities of a series of synthetic 5′-end acridine labeled phosphodiester or phosphorothioate oligonucleotides were studied using median fluorescence during flow cytometry in HL60 cells. The DNases employed were mainly endonuclease S1, the exo- and endonuclease P1, SVP (snake venom phosphodiester) and BSP (bovine spleen phosphodiesterase) [30]. It was found that the nuclease digestion, expressed as $t_{m1/2}$, proceeded with virtually identical rates for the unmodified and acridine-linked oligonucleotide, but it was about 20-fold slower for the acridine-linked oligonucleotide when BSP was used. They also found differences in T_m between the phosphodiester (PO) and phosphorothioate (PS) oligonucleotides, which decreased as the GC content was increased.

In the case of fluorescently labeled methylphosphonate (MP), PS, PO and their other analogues (4–15 mer oligonucleotides), permeation characteristics have been studied using phospholipid model membranes (liposomes) was determined by visible spectroscopy (λmax = 540 nm, in ethanol)

[5]. Transport kinetics across a lipid bilayer was found to be slow for the fluorescent MP-oligonucleotide (efflux $t_{1/2}$ of 6.2 days) compared to 5′-^{32}P-labeled oligonucleotide ($t_{1/2} = 4.4$ days).

2.2. Radiolabeling

To study the in vitro stability of radiolabeled oligonucleotides in cell-free systems, investigators have used (a) liquid scintillation counting (LSC) and (b) polyacrylamide gel electrophoresis (PAGE) followed by autoradiography, separately or in combination. Sample treatment and processing are different.

2.2.1. Liquid scintillation counting (LSC)

Temsamani et al. [31] used biological fluid (e.g. serum) or tissue (e.g. kidney) and added different concentrations of purified PS-oligonucleotide either to (a) serum or (b) to the tissue homogenates, and then processed them by proteinase K digestion followed by phenol extraction. This method is based on the extraction of the oligonucleotide from biological fluids and tissues and immobilization on a nylon membrane. The membrane-bound oligonucleotide is then hybridized with 5′-^{32}P-labeled complementary oligonucleotide and autoradiographed. The data on the film can be scanned and used to create a standard curve. A serious shortcoming is that an oligonucleotide shorter than 20 bases cannot be identified since these fragments are not retained on the membrane. Hence, this method is not suitable for oligonucleotide metabolites. If the nucleotides have undergone extensive degradation, they would lose complementarity with the 5′-labeled oligonucleotide used for detection and would obviously fail to detect the oligonucleotide and its metabolites. Antiviral use of antisense oligonucleotides has been briefly discussed using 5′-^{32}P-labeled 20-mer oligonucleotide complementary to the 5′-end of HIV RNA, the primer binding site, splicing sites, protein initiation sites and the polyadenylation signal for comparison [32]. Degradation of unmodified oligonucleotides was not found in 2 h and there were substantial amounts remaining even after 27 h in rabbit or human whole blood.

2.2.2. Polyacrylamide gel electrophoresis (PAGE)

Degradation of some oligonucleotide sequences complementary to part of the 35-nucleotide sequence which are present at the 5′-end of all trypanosome (trypanosoma brucei) mRNAs (the so-called mini-exon sequence) was studied in SDM79 medium or SPG buffers using 25% polyacrylamide–7 M urea sequencing gel [33]. 5′-end ^{32}P-labeled PO-oligonucleotides (2.5 pmol) were added to 150:1 of SDM 79 medium or SPG buffer, incubated (26°C, different duration), kept in a boiling water bath for 3 min and analyzed on 25% polyacrylamide–7 M urea sequencing gel. Autoradiographs were then quantified with a Helena densitometer. Behmoaras et al. [34] conducted a similar analysis of unmodified and 3′ acridine-linked 10-mer in wheat germ extract and rabbit reticulocyte lysate as cell-free systems. Following PAGE, bands were quantified by a densitometer. The unmodified 10-mer was fully degraded after 23 h, but the acridine substitution at the 3′-end protected the oligonucleotide from exonucleases. Wickstrom [35] studied the cell-free stability of two 15-mer γ^{32}P-labeled antisense oligonucleotides. Each 15-mer was added to 25 μl samples of rabbit reticulocyte lysate, HeLa cell cytoplasmic extract, DMEM with 5% fetal calf serum, or undiluted bovine calf serum. The samples were incubated for 1.5–2 h at 37°C and 3 μl 9 M urea, 10 mM EDTA, 0.05% xylene cyanol FF and 0.05% bromophenol blue. Samples were run on 20% polyacrylamide gel and were autoradiographed to determine degradation of the oligonucleotides. They found qualitative degradation of the oligonucleotides on the autoradiograph. To enhance stability of the oligonucleotides, capped oligonucleotides have also been used [36]. Monkey plasma was mixed with ^{35}S-labeled and 3′-, 5′- or 3′-5′-capped 20-mer phosphorothioate oligonucleotides, incubated at 37°C, and at various time intervals an aliquot was treated with proteinase K (2 mg ml^{-1}) followed by phenol-extraction and ethanol precipitation. The sample was then analyzed on PAGE, and no degradation of the oligonucleotide was observed.

Stability of LDL-associated oligonucleotide in rat serum was studied by incubating (37°C) 27 μg (LDL protein) of LDL-oligonucleotide prepara-

tion with 75 μl of freshly prepared serum from male Wister rats [37]. At regular intervals, 15 μl aliquots were diluted with 200 μl PBS and extracted with 200 μl phenol, followed by centrifugation. The aqueous layer was twice extracted with 200 μl of ether. To 175 μl of the aqueous phase, 175 μl of 3 M sodium acetate, 10 μl tRNA and 800 μl of ethanol was added. After incubation for 30 min at −80°C, the samples were centrifuged. The supernatant was discarded and the precipitate was further dried on a Speed-Vac Evaporator. The samples were then analyzed by electrophoresis (using 7 M urea–19% polyacrylamide) gel followed by autoradiography. They found that cholesterol linked oligonucleotide remained essentially intact for 5–10 min in rat serum. The control was degraded more rapidly. However, the control oligonucleotide contained the linker moiety that relatively protected against degradation. Underivitized oligonucleotide was degraded more than 50% within 1 min.

To study the degradation of oligonucleotide in various commonly used biological systems for antisense inhibition studies, a 15-mer pentadecadeoxynucleotide complementary to residues 9–23 of human *c-myc* mRNA was labeled at the 5′-end with ^{35}S. The labeled oligonucleotide was added to 18 μl aliquots of the biological samples i.e. rabbit reticulocyte lysate, HeLa cell postmitochondrial extract, RPMI-1640 with 10% fetal bovine serum, undiluted fetal bovine serum, adult human serum and rat cerebrospinal fluid. The samples were incubated at 37°C, and 3 μl aliquots were removed at various time points. Reactions were terminated by the addition of each aliquot to 3 μl of 80% formamide, 0.1% xylene cyanol FF, 0.1% bromophenol blue and subsequent freezing. The aliquoted samples were analyzed by PAGE, followed by soaking in Fluorohane (RPI), drying on 3 MM Whatman paper and autoradiography at −80°C. The bands were quantified by laser scanning densitometry and semilogarithmic plots and were fit using Sigma Plot (Jandel). It was observed that in all the systems (including a control with water), a significant amount of the labeled oligonucleotide phosphorothioate remained in the wells. Slow and steady degradation of the oligonucleotide was detected in all the systems. The longer exposed autoradiograms displayed ladders of shorter oligonucleotides, as would be expected for 3′-exonuclease activity in all systems except for the control. They found that the half-life of the oligonucleotide spanned 7–19 h at 37°C, sufficiently long for biological experiments [38].

Combination of LSC and PAGE has also been used by some investigators [39–41]. Permeation characteristics of ^{32}P-labeled methylphosphonate, phosphorothioate, phosphodiester and their other analogues (4–15 mer) have been studied through biological membranes using phospholipid model membranes (liposomes) [39]. The oligonucleotides were antisense to a 5′ splice site in human β-globin gene or human MDR-1 gene (AUF initiation codon). Binding (adsorption) and efflux of oligonucleotides to and from lipid membranes were analyzed using LSC after resuspending the washed lipid pellet in 1 ml PBS. They used titratiated sucrose and glucose for comparison. Similarly, the amount of radiolabeled oligonucleotide retained within the liposomes (pellet) was determined by LSC, and that of the fluorescent species by visible spectroscopy ($\lambda max = 540$ nm in ethanol). The biological stability of oligonucleotides both within liposomes and in the efflux medium was assessed by PAGE (15% polyacrylamide containing 7 M urea). In case of entrapped oligonucleotides, the liposomes were permeabilized with Triton X100 (0.5% v/v) to free entrapped oligonucleotides prior to analysis by PAGE [39] at the end of efflux study (8 days). The PAGE analysis showed that the oligonucleotide (5′-end ^{32}P-labeled 14-mer unmodified PO-oligonucleotide) remained stable both in and outside the liposomes over the 8 day period.

Gottikh et al. [40] used sequences of eight β-nucleotides flanked at 3′- or 5′-ends by nuclease resistant α-oligonucleotides, and complementary to the translation initiation site of the *pim* protooncogene mRNA, as substrates for RNase-H in three biological media, i.e. human plasma, calf serum and mouse fibroplast lysate. 15–24 mer Oligonucleotides were used. The synthetic phosphodiester αβ-oligonucleotides were 5′-end labeled with ^{32}P. To protect the labeled oligonucleotides from phosphatase activity, either

(1) radiolabeled 5′-phoshates of oligonucleotides were converted into their ethyl esters using 1-ethyl-3,3′-dimethylaminopropyl carbodiimide, or (2) the oligonucleotides were labeled internally by chemical ligation in the presence of the 5′-end-labeled oligonucleotide. Plasma was obtained by centrifugation of heparinized whole human blood (10 000 × *g*, 5 min). The murine DUNNI fibroblast lysate was prepared by suspending 4×10^6 cells in 1 ml water, maintained at 0°C for 10 min, and centrifuging at 10 000 × *g* for 10 min. The labeled oligonucleotide was added to the biological media (total volume 50 μl, 10^5–10^6 cpm total activity). At various time points, 5 μl aliquots were removed, and the reaction stopped by extraction with phenol–chloroform–iso-amyl alcohol (25:24:1). The oligonucleotide was precipitated from aqueous samples by acetone containing 2% $LiClO_4$, dried, and dissolved in 4 μl formamide–water (4:1), 0.01% bromophenol blue, 0.01% bromophenol blue and 0.01% xylene cyanol. Samples were electrophoresed on 20% polyacrylamide–7 M urea gel. The radioactivity products were visualized by autoradiography. Degradation of oligonucleotides was quantified by LSC of the radioactive bands corresponding to intact and degraded oligonucleotides once these bands were removed from the gel. Goodchild et al. [41] conducted similar studies in freshly drawn rabbit or human blood using 200 nmol of ^{32}P-labeled oligonucleotide. However, they extracted the aliquots using equal volume of phenol saturated with water followed by phenol–chloroform–iso-amyl alcohol (25:24:1). Samples of the aqueous layer were used for scintillation counting, and for electrophoresis in 50 mM tris borate (pH 8.0) on 20% polyacrylamide gels containing 8 M urea.

2.3. High performance liquid chromatography and other methods

High performance liquid chromatography (HPLC) is the most convenient and versatile method for the purification of synthetic DNA. Brown and Brown [42] have described HPLC hardware, and maintenance of the instruments. Strong anion-exchange chromatographic purification along with reverse-phase HPLC can yield quite pure DNA. The separation of oligonucleotide by the HPLC and capillary electrophoresis (CE) can be further analyzed by atmospheric pressure ionization (API) and mass spectrometer (MS) as an ultimate detection technique. This can be achieved by coupling the separation device (HPLC or CE) with the detection device (MS with API) [43].

Hatestam and Pinkerton [44] developed the concept of direct HPLC analysis of biological sample using an internal-surface reversed phase (ISRP), followed by conventional reversed-phase procedures. In the materials used, the inner surface of the silica bonded particle is hydrophobic and the outer surface is hydrophilic. Hence, the macro-molecules too large to penetrate into the pores are quickly eluted in aqueous solvent. Small molecules penetrating the pores are subjected to partition chromatography. In summary, protein, salts and other hydrophilic compounds (e.g. amino acids) contained in the cell culture media are first eliminated before hydrophobic compounds are eluted by the conventional reversed-phase procedure.

Pompon et al. [45] believed that there are several disadvantages to precise analysis of the stability of oligonucleotides in biological systems by electrophoretic separation and autoradiography of the ^{32}P-labeled oligonucleotides or their metabolites. The use of only purified enzymes, or because gel electrophoresis analysis of ^{32}P-labeled oligonucleotides allowed only the detection of 5′-labeled fragments, were thought to be the reasons. Secondly, in conventional HPLC, extensive sample preparation poses a limitation to studying the kinetics of oligonucleotide degradation. Hence, an alternate procedure to readily determine rate and mechanism of oligonucleotide degradation in cell culture media by direct analysis on reversed-phase or ion-exchange HPLC was reported. 19-mer and 12-mer oligonucleotides were used to evaluate the analytical approach and stability in the cell culture medium (containing 10% heat inactivated serum and antibiotics and stored in aliquots (−20°C) till used as biological samples). The synthetic oligonucleotide was mixed and diluted with the biological samples before analyzing by HPLC.

The internal surface reversed phase (ISRP) with HPLC procedure was used to analyze the biological fluid (serum) without any pre-treatment or internal standard, using a C8-ISRP precolumn followed by a C18-reversed-phase or an ion-exchange column. HPLC was performed on a Waters-Millipore instrument using a precolumn (C8, 10 μm, 4.6 × 10 mm), a reversed-phase column (C18, 3 μm, 4.6 × 100 mm), and an ion-exchange column (Ultranucleotide PVDI 4000-05, 10 μm, 4.6 × 100 mm). A similar approach was used by Morvan et al. [46] to evaluate the comparative stability of seven different species of 12-mer oligonucleotide analogues, complementary to HIV-1 tat gene in the culture medium (RPMI-1640 with 10% heat inactivated (56°C, 30 min) fetal calf serum), using the HPLC technique of Pompan et al. [45] The culture medium was first purified to remove all extraneous substances except the oligonucleotides and its metabolites on a short pre-column ISRP column (Ultrabiosep C8, 10 μM, 4.6 × 10 mm), using buffer A (20 mM potassium phosphate, 0.2 M potassium chloride, pH 6.0). Next, this column was connected to another column which combined ion-exchange and reversed-phase properties (Ultranucleotide 4000-10 10 μm, 4.6 × 100 mm). The oligonucleotide–metabolites from the first column were transferred to the second column by buffer B (20% acetonitrile v/v in 20 mM potassium phosphate, 0.2 M potassium chloride) in 3 min by an actuating valve. The transferred oligonucleotide–metabolites were then chromatographed according to their charge and lipophilic contents when the ionic strength of the solvent was increased by the linear gradient of B–C (as B, except 0.4 M potassium chloride). They have presented HPLC analysis plots of the crude sample after 2 h incubation (as absorbance versus retention time and also relative concentration of different metabolites versus incubation time). They determined the half-life ($t_{1/2}$) of oligonucleotides based on the disappearance of the starting material in the culture medium. All PO-oligonucleotides were half-degraded in 1 h against PS-oligonucleotides, which were most resistant to >10 days (for α-anomeric PS-oligonucleotides). For β-anomeric configuration, a progressive hydrolysis from the 3′-end was observed.

Serum stability of oligonucleotide was studied by Sands et al. [47]. About 1 μCi of ^{3}H-labeled oligonucleotide was mixed with 1 μM cold (unlabeled) oligonucleotide in non-heat inactivated mouse serum and incubated at 37°C for different times. Oligonucleotide and its metabolites were extracted twice by equal volume of phenol–chloroform (1:1, v/v) by centrifugation (10 000 × *g*, 30 min). The pooled aqueous layer was analyzed by HPLC. The chromatogram showed the ultimate monomer metabolites and full size (20-mer) oligonucleotides, and other intermediate metabolites were also detected by HPLC. The apparent halflife of the 20-mer species was approximately 30 min in serum.

Tidd and Warenius [48] studied the stability of 16-, 18- and 20-mer methylphosphonate oligonucleotides in McCoy's 5A tissue culture medium containing 15% heat inactivated fetal calf serum in the presence or absence of exponentially proliferating HT29/5 cells. At various incubation times, aliquots of the medium were removed, mixed with EDTA and 100 mM TEAA (pH 7.0), and purified on a C18 Sep-Pak. Washing was done with 10 ml, 100 mM TEAA and 5 ml 5% acetonitrile, followed by 10 ml water. Eution was done with 1.2 ml 30% acetonitrile in water, and concentrated on a Speed Vac before analyzing by SAZ-HPLC. The peaks were identified by their retention times. These oligonucleotides, coupled with methylphosphate diester or monoester end groups at the 3′-end or both 3′- and 5′-ends and their respective piperiding hydrolysates, were incubated with 0.02 U phosphodiesterase I from *Crotalus adamanteus* venom in tris-buffer. Aliquots at different times of incubation were mixed with EDTA and acetonitrile, and applied to C18 Sep-Pak cartridges, washed with 30% acetonitrile, and the combined effluents were vacuum dried and analyzed by reverse phase HPLC on an Aquapore RP-300 column [48]. 20-mer All PO-oligonucleotide showed complete degradation within 2 h of incubation against partial degradation of chimeric oligonucleotide having 2-methyl phosphonates at either ends of the oligonucleotide but having the same base sequence as determined by HPLC peaks. They concluded endonucleolytic cleavage as the first step in degradation. In case of chimeric

oligonucleotide, 44% of the original oligonucleotide was present at 4 h. PO-oligonucleotide did not remain intact in 4 h, whereas 17–30% of the initial concentration of the chimeric oligonucleotide was detectable at 22 h.

A method to recover input oligonucleotide from cell culture media was proposed by Marcus-Sekura [49]. Recovery of test oligonucleotide–methyl phophonates from CV-1 cell culture media was done by drying the medium in a Speed-Vac (Savant), mixing with water (2–4 ml), and then with an equal volume of ethanol. After thorough vortexing, the mixture was allowed to stand at room temperature for 4 h before low speed centrifugation at room temperature and subsequent removal of the supernatant, which was subjected to reversed-phase HPLC (yielding $\approx$ 50% recovery of the input oligonucleotide).

In a cell free system, Bourque and Cohen [50] proposed an elegant method of generating single-blind spiked sample data, with an internal standard, to quantitate sample oligonucleotide. Human serum (250 μl) was spiked with 10- and 25-mer phosphorothioate oligonucleotides (250 ng of each), processed with proteinase K, phenol–chloroform–iso-amyl alcohol, dithiothreitol (DTT), diethyl ether and formamide, etc. and finally injected on fast anion-exchange column HPLC. The standard curve was developed from the known concentrations of 25-mer spiked into serum containing 125 nm of the 10-mer (as the internal standard), and processed as above. The resolution of PS-oligonucleotide of 25-mer, and smaller fragments, has been accomplished in less than 4 min by fast-anion exchange chromatography.

2.4. Summary of measurement of oligonucleotides in cell free systems

To understand the specificity of an antisense oligonucleotide for target proteins or mRNA, and to compare biological stability of these oligonucleotides, the cell free systems provide a convenient and economic approach. Fluorescently labeled oligonucleotide, apparently, provides technology as compared to radiolabeling techniques. However, the interpretation of different degrees of quenching of fluorescence signal in biological milieu poses an apparent limitation over radiolabeling techniques. Both TLC and PAGE techniques, followed by densitometry, have been used for quantification of oligonucleotides or their metabolites in case of both fluorescent and radiolabeled oligonucleotides. The modified HPLC techniques [45,46] seem to provide more reliable measurement of oligonucleotides in biological systems. This method can provide reasonable limits of detection (20–30 pmol), resolution (3–5 bases) and recovery (85-90%) over the other two methods, though at the initial capital cost of the instrument (Table 1).

3. Cellular uptake of oligonucleotides

The cell uptake of oligonucleotides has been evaluated in order to demonstrate potential availability of the oligonucleotides for the designated target molecules. Studies should embrace issues of dose-dependence, time course, sub-cellular distribution, concentration achieved within cellular compartments, and the integrity of the oligonucleotides at putative target sites. Limitations in all cell uptake studies include the identification of oligonucleotides in cells which does not ensure oligonucleotide bioavailability to the designated target. Uptake studies should be accomplished by mechanistic studies such as those of Giles et al. [51], in which the recovery of predicted fragments of mRNA was addressed. Readers interested in studies on cell-toxicity or cell growth inhibition by oligonucleotides are directed to references [13,52]. Only those studies wherein the fate of input oligonucleotides in cell-culture have been addressed are reviewed here.

3.1. Fluorescence

Studies of fluorescently labeled oligonucleotides have been conducted using a number of approaches, such as fluorescence microscopy [7,53], confocal microscopy [54], spectrofluorometry [55], video microscopy [56], fluorescence densitometry [56] and flow and cytometry [30,55,57–61].

Table 1
Cell free analysis of oligonucleotides

Method	Sample/substrate	Conclusions	Reference
Fluorescence			
Oxazolapyri-do-carbazole	NIH3T3 cell extract	α-anomar of C1-sugar more stable compared to β-anomer	[29]
Acridine	Wheat germ extract and rabbit reticulocyte lysate	Acridine Protected degradation	[34]
Radiolabeling			
[^{32}P]dATP	Liposomes	Cellular transport, $t_2 = 6.2$ days	[39]
[^{32}P]dATP	Serum and tissue homogenate	Oligonucleotides longer than 20-mer can be detected	[31]
[^{32}P]dATP	Rabbit and human whole blood	No degradation of oligonucleotides in 2 h	[32]
[^{32}P]dATP	Culture media	Oligonucleotide degradation studied	[33]
[^{32}P]dATP	Rabbit reticulocyte lysate, HeLa cell cytoplasmic extract, fetal and bovine serum	Oligonucleotide degradation studied	[35]
[^{35}S]dATP	Monkey plasma	Oligonucleotide degradation	[36]
[^{32}P]dATP	Rat serum	Oligonucleotide degradation	[37]
[^{35}S]dATP	Rabbit reticulocyte, HeLa cell postmitochndrial extract, RPMI 1640; fetal bovine and human serum and rat cerebra-spina fluid	Oligonucleotide degratation half-life 7–9 days	
[^{32}P]dATP	Human plasma, calf serum and mouse fibroblast lysate	Oligonucleotide degradation	[40]
[^{32}P]dATP	Rabbit and human blood	Oligonucleotide degradation	[41]
HPLC			
ISRP-	RPMI-1640	Oligonucleotide degradation	[46]
Paired-ion	Mouse serum	Half-life 30 min	[47]
Reverse phase	Tissue culture	PO-oligonucleotide more stable than PS-oligonucleotide	[48]
Reverse phase	Culture medium	50% oligonucleotide recoery	[49]
Anion exchange	Human serum and urine	20 ppb PS-oligonucleotide detected, 5 base resolution	[50]

Stein [62] proposed endocytosis followed by pinocytosis as the possible mechanism of uptake of oligonucleotides. HL60 and Du 145 cells were used to study internalization of FITC labeled PO and PS oligonucleotides by quantitating the quenching of the FITC signal of oligonucleotides in lysosomes, apparently produced by endocytosis followed by pinocytosis. The fluorescence intensity of FITC-labeled oligonucleotide in lysosomes was maintained by neutralizing the pH gradient within the cell by the addition of monensin. The internalization of the PS and PO oligonucleotides was found in two compartments within the cell, thus representing an endosomal pathway of oligonucleotide intake by cells. About 65% of the PO oligonucleotide (15- or 20-mer) was in the shallow compartment, and 80% of the PS oligonucleotide (15- and 28-mer) was in the deep compartment.

Fluorescein-labeled ISIS 1570 (18-mer) was found, having increased localization within nuclei, with the use of a cationic lipid preparation, *N*-[2,3-dioleyloxy)propyl]-*N*, *N*, *N*-trimethylammonium chloride (DOTMA) than otherwise in human umbilical vein endothelial cells (HUVEC) grown on fibronutin-coated glass microscope slides [53]. Cells were treated with FITC-labeled oligonucleotide in the presence or absence of DOTMA. At indicated times, cells were washed 4 times with D-PBS and fixed with 2% formaldehyde in D-PBS for 20 min at 25°C. After fixation, cells were washed three times with D-PBS and mounted in glycerol mounting solution (10 mM phosphate, pH 8.5, 150 mM NaCl, 70% glycerol).

The subcellular localization of the FITC-labeled oligonucleotide was determined by fluorescent microscopy, using a Nikon Optiphot-2 fluorescent microscope. Leonetti et al. [7] used a similar approach to study 15-mer oligonucleotides (phosphodiester or phosphorothioate derivatives) conjugated at their 5′-ends to FITC (fluorescein isothiocyanate), TRITC (tetramethyl-rhodamine isothiocyanate), or 7-amino methyl coumarin hydroxy succinate ester (AMCA). They microinjected the oligonucleotides into the cytoplasm of rat embryo fibroplasts (REF-52), and their cellular distribution was monitored by fluorescence microscopy in fixed or non-fixed cells. After injection, cells were incubated (37°C, specified times), fixed in ethanol–acetone, mounted, and photographed using an Axiphot Zeiss Photomicroscope. The study of the stability of oligonucleotides in nuclei showed that the fluorescence remains at a maximum level for a relatively short period of time and decreases thereafter (undetectable 6–10 h later), which could suggest degradation of oligonucleotide.

It has been shown [56] that the phosphodiester antisense oligonucleotides (anti-rev and anti-tat), encapsulated in immunoliposomes, were 100 fold more effective in acutely and chronically HIV-infected CCRF-CEM cells, (a T lymphoblastoid cell line). For fluorescence-activated cell sorting (FACS) analysis of input oligonucleotides, liposomes were prepared with the aqueous phase containing 40 mM purified carboxyfluoresceing (CF). The final concentration of the CF was determined by spectofluorometric measurements.

Chin et al. [56] studied the intracellular transport and fate of fluorescent oligonucleotides (28-mer)—PO, PS and methylphosphonate (MP)—in African Green monkey-derived epithelial cells and primary human fibroblasts using time-lapse video microscopy, and fluorescence densitometry. The oligonucleotides used were complementary to the rev mRNA of human immunodeficiency virus type 1, (HIV-1). The data presented help understand the transport and accumulation of exogenous nucleic acids in mammalian nuclei, and the assay was potentially useful in testing the efficacy of oligonucleotides designed as therapeutic agents. The fluorescence was calculated as the ratio of nuclear to total fluorescence, after subtracting the background and correcting for shading.

Many investigators have used flow cytometic analysis to determine cellular intake and distribution of synthetic oligonucleotides. In the uptake study of acridine labeled oligonucleotides, (i.e. dT_7, dT_{12}, dT_{15} and dT_{20} by HL60 cells), it was found that the shorter oligonucleotides were more rapidly taken up than the longer ones, and the diester over phosphorothioates [30]. The oligonucleotides were incubated with HL60 at (0.2–0.5 μM final concentration) in RPMI-1640 medium containing 10% fetal calf-serum and antibiotics. At various time points, 10^5 cells were removed, washed 3 times, and analyzed using flow cytometry by exiting intracellular acridine (488 nm, 300 mW). Based on median fluorescence, the nuclease sensitivity of 5′-Acr-dT_{15} was also evaluated. They found temperature dependence of cellular uptake, prompting a conclusion of receptor mediated internalization of these oligonucleotides. Besides the myeloid cell line (HL60), human Burkitt lymphoma cell line Daudi, the acute lymphoblastic leukemia T-cell line MOLT-4, and murine myeloid line DA1 were used by Loke et al. [57]. Acridine labeled oligonucleotide was found accumulated intra-cellularly in a temperature dependent manner. Besides structural specificity of the oligonucleotide uptake, they also studied the cellular uptake saturability and specific inhibitability by unlabeled oligonucleotide. Brieg et al. [58] studied the uptake of an internally labeled [γ^{32}P]dATP at the 5′-end of a 12-mer ligated with a 14-mer and a fluorescein (FITC) labeled 24-mer by cultured murine spleen and lymph node cells using flow cytometry. Internal labeling, indicates that the [γ^{32}P]dATP was situated internally, and not at the 5′- or 3′-end of the oligonucleotide. Lymphoid subpopulations were distinguished by flow cytometry and staining with antibodies to cell-surface markers. About 5% of fresh lymphoid cells take up substantial amounts of oligonucleotides. They found that the percentage of B cells internalizing oligonucleotides is dependent on the time duration of preculture before incubating with oligonucleotides. The oligonucleotide uptake was quite heterogeneous among cultured cells and was mitogen inducible. The data presented may be

important for design and interpretation of in vitro experiments directed towards antisense therapy.

Spiller and Tidd [59] instead used chimeric oligonucleotides. They tagged fluorescein reporter groups at the 5′-termini of chimeric oligonucleotides with terminal nonionic methyl-phosphonate analogue sections and internal phosphodiester regions, all-phosphodiester, and all-methylphosphonate oligonucleotides c-myc (15-mer) and studied the uptake kinetics in human leukemia MOLT-4 cells. Standard curves for flow cytometric quantitation of oligodeoxynucleotides were derived by using APS-Hypersil 5 micro 'Hyperspheres', to which known amounts of flourescein tagged all-phosphodiester oligonucleotides had been irreversibly bound. Calibration suspensions were prepared simply by mixing known amounts of oligonucleotides with known numbers of the spheres (the HPLC support material), which was determined by counting samples on a Model ZM Coulter Counter. UV spectrophotometry was used to demonstrate that all added oligonucleotide had been bound to the spheres, and that no material was eluted by washing with PBS. The fluorescence yield of the spheres was stable for several weeks at 4°C or for months at −20°C in the PBS. They prepared a standard curve of a flow cytometer florescence versus oligodeoxynucleotide standards response curves. Tidd [51] presented a technique of enhancing cellular uptake of nuclear localization of chimeric PO oligonucleotide having methylphosphonates at both ends by using streptolysin to permeabilize the plasma membrane. Using the RT-PCR method to demonstrate antisense specific reduction in the target mRNA, the chimeric oligonucleotide experiment also showed the RNaseH mediated cleavage of targeted mRNA at the heteroduplex site. It was, therefore, an indirect apporach to demonstrate an increase in cellular uptake of the chimeric oligonucleotides. The uptake kinetics showed that the MP oligonucleotide uptake was non-saturable, and initial rates were directly proportional to extracellular concentration in contrast to PO oligonucleotides.

Leonetti et al. [60] investigated the internalization pathway of poly(L-lysine (PLL)) conjugated and fluorescently labeled oligonucleotides (16-mers) in L929 cells. The oligonucleotides were fluorescently labeled with carboxyfluorescein *N*-hydroxysuccinimide ester, and then linked to PLL through an *N*-morpholine ring by periodic acid oxidation and borocyanohydride reduction of the 3′-end ribose. For flow cytometry analysis, L929 cells (2×10^5/well in a 24-well plate) were incubated at 37°C with oligonucleotides (1 μM) linked or unlinked to PLL. At various time points, 20 U ml^{-1} DNase I was added in the culture medium for 10 min at 37°C. The cells were then washed twice with PBS, scraped from the plates with PBS and 10 μM EDTA, and stored in PBS, 10 mM EDTA, 3% (v/v) paraformaldehyde prior to analysis on a fluorescence activated cell sorter. The uptake was maximal between 4 and 9 h after addition to the culture medium, and later fluorescence decreased rapidly. Uptake of fluorescence PLL was faster, and greatly increased as compared to unconjugated oligonucleotides.

Both flow cytometry and fluorescence microscopy were used to study fluxes (at 37°C in CO_2 air incubation) and localization of a fluorescein conjugated (i.e. *N*-nonomethoxy-tritylaminohexyl) derivative of phosphorothioate oligonucleotide (28-mer) in hematopoietic cells (H9) [61]. The cellular uptake of oligonucleotide was halted by excess (4 ml) fresh medium, followed by centrifugation at 4°C. Cells were resuspended in fresh RPMI-1640 medium supplemented with bovine serum and analyzed immediately on the flow cytometer. By a competitive uptake study, they found that the derivitized material was better able either to enter or stay within the cell.

There are a few limitations to the flow cytometric analysis of oligonucleotide determiantion which have been discussed by Marti et al. [61]. Since flow cytometry measures the number of derivitized molecules per cell, the cell column was calculatd to determine intracellular concentration. This was done by measuring cell-radii by light microscopy and a calibrated reticule, assuming that the cells are spherical ($v = 4/3\ \pi r^2$). From the standard curve using fluorescein fluorescence and the estimated cell volume, the mean channel fluorescence of 100 corresponded to an internal concentration of 0.5 μM (assuming that all the

oligonucleotide was internally isotropically distributed). Qualitative estimation of the fluorescent oligonucleotide in the HeLa and H9 cells was made by using differential interference contrast (Nomarski optics) and fluorescence microscopy. Since flow-cytometry experiments frequently do not distinguish intracellular from surface-bound material, the assumption of a rapid surface adhesion for 5 min or so incubated fluorescence would require a correction of about 20–25%, which seems overly severe. A further difficulty in interpreting the fluorescence data in terms of averaged intracellular concentrations is the unknown extent of quenching of the fluorescent chromophore within the cell due to different effects of various cellular proteins, besides the fact that fluoresceing fluorescence is markedly reduced at low pH values as might occur in endosomes.

3.2. Radiolabeling

Radiolabeling of oligonucleotide can be done by using ^{14}C, ^{35}S, ^{32}P, ^{3}H or ^{125}I radio-isotopes. For specific procedures to do this, readers are directed to the literature. Such labeled oligonucleotides have been used both for in vitro and in vivo studies. Detection or determination of the labeled oligonucleotide can be done in two ways, i.e. by LSC and PAGE, followed by autoradiography.

3.2.1. LSC

In general, cell associated oligonucleotide or its internalization has been studied by adding radiolabeled oligonucleotide to cell-culture for a specified period followed by centrifugation and washing with culture medium before counting the radioactivity by LSC. Following this approach, a ^{32}P-labeled 15-mer oligonucleotide was used by Bellan et al. [63] to specifically inhibit c-myc protein expression in human T lymphocytes; 5′-^{32}P-labeled pTn ($n = 8$, 0, 10 and 16) oligonucleotides were studied by Yakubov et al. [64] using mammalian cells, L929 mouse fibroblasts, and Krebs 2 ascites carcinoma cells grown in mice. Tritium labeled oligonucleotide methylphosphonates (9-mer) were studied by Miller et al. [65] in mammalian cells. Internally labeled [$\gamma^{32}P$]dATP at the 5′-end of a 12-mer ligated with a 14-mer oligonucleotide was studied by Krieg et al. [58] in murine spleen cells. In the latter case, the cell pellet obtained after centrifugation was dried onto glass fibre filters before scintillation counting. 5′-$\gamma^{32}P$-labelled and 5′-end modified by covalent attachment of a phospholipid [triethylammonium 1,2-di-*O*-hexadecyl-*rac*-glycero-3-H-phosphonate] 10–20 mer oligonucleotides antisense to different initiation codons of different proteins of VSV (vesicular stomatitis virus) was similarly studied by Shea et al. [66] in L929 (ATCC). They found inhibition of VSV protein synthesis with such conjugated oligonucleotides.

Wu-Pong et al. [67] adopted little variation in processing between cell culture treatment of oligonucleotide followed by LSC. Under different processing or treatment conditions of the Rouscher Red $5-1.5$ cells, 10^4-10^5 cpm ml^{-1} ^{32}P-labeled oligonucleotide mixed with unlabeled oligonucleotide resulting in a final concentration of 0.1–10 μM was added to the cells. At each sampling time, a 100 μl sample was centrifuged along with 100 μl 2:7 dionyl phthalate–dibutyl phthalate in a 0.4 ml tube. All samples were frozen at −70°C, pellets were clipped off, dissolved in 100 μl of 0.1% SDS, and counted in 4 ml of scintillation cocktail. A similar study was done by Wichstrom et al. [68] in HL-60 by 5′-^{35}S-labeled 15-mer c-myc antisense oligonucleotide. For each time point, 5×10^5 cpm of 5′-^{35}S-labeled oligonucleotide was added to 4×10^6HL-60 cells in 0.5 ml of RPMI-1640 with 10% heat-inactivated fetal bovine serum. After incubation, cells were sedimented (3 min, $15000 \times g$), the supernatant was removed, and the cell pellet was washed once with 0.5 ml of phosphate buffered saline (10 mM Na_2HPO_4, pH 7.4/150 mM NaCl) and sedimented again. The cell pellet was lysed in 0.1 ml tris buffered saline (10 mM Tris–HCl, pH 7.4/150 mM NaCl) with 1% sodium dodecyl sulphate, and then extracted with 0.1 ml phenol. The aqueous phase was removed and the phenol phase extracted with 0.1 ml of water. Aliquots of the combined aqueous extracts, cell wash, and culture-medium supernatant were analyzed by LSC. The percent oligonucleotides taken up by the cells

was calculated by dividing counts in the combined aqueous phase of the cell-pellet extract by the total of the counts in the pellet, cell wash, and culture-medium supernatant [68]. Similar intermediate processing steps were followed by Vasanthakumar and Ahmed [69] using 5′-[γ^{35}S]ATP labeled in K562/III cells. After incubation and centrifugation, cells were lysed with SDS and then extracted with 0.1 ml phenol. The aqueous phase was removed and the phenol phase extracted with 0.1 ml water. Aliquots of the combined extracts, cell wash, and the culture medium supernatant fluid were analyzed for radioactive counts (LSC). They found a time dependent increase in label associated with these cells. Stability of these oligonucleotides was studied by using 20% polyacrylamide–7 M urea sequencing gel, and it was found that the oligonucleotides were intact for 24 h and that little degradation was observed at 48 and 72 h. Radioactivity retained by the washed cells was compared with that left in the medium. There was a time dependent increase in label associated with K562/III cells, however the radioactivity did not reach saturation.

Jaroszewski et al. [70] harvested cells by trypsinization, and isolated them by centrifugation, to study ^{35}S-labeled phosphorothioate oligonucleotides (i.e. S-dC_{14} and S-dC_{28}) in two cell lines, MCF-7 WT, MCF-7 ADR. Further, lysing, various salt precipitation and differential centrifugation techniques were used to separate various cell components. The radioactivity of cells and individual fractions was determined by liquid scintillation counting. The cell harvest was filtered (0.4 μm) before counting. The cellular uptake of the radiolabeled phosphorothioate oligonucleotide was only 1.6–2.2% of the total amount added to the medium. The cytoplasm showed $\approx$ 60% total radioactivity, and the nuclei the least (5–6%).

In a study of the comparative ability of different oligonucleotide analogues of identical sequences to inhibit expression of chloramphenicol acetyl transferase (CAT) activity and cellular uptake in CV-1 cells, the latter were incubated with ^{32}P-labeled oligonucleotide–methylphosphonate (6.56×10^6 cpm) at 37°C for varying time points. Duplicate 100 μl aliquots (containing $\sim 10^7$ cells) were removed at specific times, layered on the surface of 500 μl of pre-chilled silicone oil, and then centrifuged. The cell pellet at the bottom was removed, briefly inverted on absorbent paper to drain, transferred to a scintillation vial, and counted [49]. The phosphorothioate analogues were twice as potent as methylphosphonates, and four times more so than normal phosphodiester analogues.

ISIS 1047 (21-mer), both internally labeled and end labeled with ^{32}P, was used for an uptake study in HeLa cells. By measuring cell associated radioactivity as percent of total input using LSC, it was found that ^{32}P-end labeled ISIS 1047 had twice the cell associated radioactivity than that of ^{32}P-internally labeled oligonucleotide [52]. Biochemical and cellular pharmacology of a ^{32}P-labeled 20-mer antisense p. 120 phosphorothioate oligonucleotide, ISIS 3466 with human amelanotic melanoma (LOX) tumor cells, has been studied in the presence of DOTMA (10 μg ml^{-1}), which increased 100-fold higher concentration of the oligonucleotide in LOX cells [71].

3.2.2. PAGE

PAGE analysis, followed by autoradiography and densitometry, is done to study the in vitro or in vivo stability of oligonucleotides. Following this approach, a 5′-^{35}S labeled 15-mer methylphosphonate oligonucleotide stability was studied in K562/III cells [69]. Following incubation for various times up to 72 h, the cells were centrifuged (15 000 × g, 3 min), the pellet was washed (0.5 ml PBS), lysed with SDS, and then extracted with 0.1 ml phenol. The aqueous phase was removed, and the phenol phase extracted with 0.1 ml water. These extracts (aliquots of the combined aqueous phases and culture medium supernatant) were lyophilized, electrophoresced, and autoradiographed. Oligonucleotides were intact for 24 h, and a small degradation was observed at 48 and 72 h [69]. The concentration of the oligonucleotides in liposomal preparations used for treating acutely and chronically infected CCRF-CEM cells, a T lymphoblastoid cell line, was determined by trace labeling with [γ^{32}P]ATP at the 5′-end using T4 polynucleotide kinase [51] followed by PAGE analysis. The PO oligonucleo-

tides (anti-rev and anti-tat) thus encapsulated were 100 fold more effective in CCRF-CEM cells.

Penetration of oligonucleotide into the cytoplasm and nuclei of PC12 cells (the NGF responsive cell line, rat pheochromocytoma) was studied [72] by incubating 100 μl of 2×10^5 ml^{-1} of PC12 with 50 ng ml^{-1} of NGF and 50 μM oligonucleotides (18–21 mers) labeled at the 5′-end with [^{32}P]dATP. Following incubation, cells were centrifuged (3 min, 2000 × *g*), washed with PBS, and lysed in 50 μl RSB, and dissolved in 0.1% SDS solution. The fractionated nuclei and cytoplasmic fractions were treated with enzymes, extracted with phenol–chloroform (1:1) and chloroform–iso-amyl alcohol (24:1), and ethanol precipitated before they were electrophoresced on 12% polyacrylamide gel containing 7 M urea. Within 30 min, the penetrated oligonucleotides in the cytoplasm showed degradation. By 7 h, most of the oligonucleotides had penetrated a nucleus from cytoplasm. It was found that only 1% of the oligonucleotide penetrated the cells. Using a similar method, stability of the ^{32}P-labeled 16-mer oligonucleotide internalized by L929 cells (mouse fibroblasts) [64], ^{35}S-labeled oligonucleotide by HL-60 cells [73], ^{32}P-labeled PS-oligonucleotides on several cell lines [25,26,34,64] has been studied using denaturing gel electrophoresis (generally 20% PAGE). Various end modified (3, 5′,3′–5′) or 'capped' phosphodiester or phosphorothioate oligonucleotides 5′-end ^{32}P-labeled oligonucleotides [74] were studied for nuclease stability on HeLa cells. The intracellular fate of the labeled oligonucleotide was determined by harvesting the cells by trypsinization. Both supernatant and harvested cells were extracted with equal volume of phenol saturated with buffer. The phenol extracted sample was precipitated and resuspended in 9 M urea in 2 × TBE (0.2 M HCl, pH 8.3, 0.2 M sodium borate, 4 mM EDTA). The samples were analyzed by gel electrophoresis, on 20% polyacrylamide containing 7 M urea. After PAGE an X-ray film was exposed (autoradiographed) either at room temperature or at −70°C overnight [67] in order to visualize the labeled oligonucleotide bands (which locates either the full size (parent) molecule or its metabolites). Between 60–120 min, 28% of oligonucleotide in the medium was degraded to smaller metabolites or monomer. More than 50% of the oligonucleotide remained intact in the medium and the oligonucleotide degradation within the cell was not significant during this period.

Geselowitz and Neckers [75] studied oligonucleotide binding, internalization, and intracellular trafficking using a photoactive and radioiodinated (^{125}I) crosslinker (the Denny-Jaffe reagent) in HL60 cells in culture. The oligonucleotides containing pendent amine groups at either the 5′- or 3′-end (using either AminoLink2 (Abi) or 5′-Aminomodifier C (Glen Research) during oligonucleotide synthesis), were conjugated to this agent, added to HL60 culture, and photocrosslinked to associated proteins which were analyzed electrophoretically. When the conjugate (crosslinker and oligonucleotide) is exposed to UV light, the aryl azide group of the crosslinker crosslinks to any cellular component in close proximity (≈ 16 Å). The complex can be chemically cleaved at the azo linkage, and the iondinated portion of the crosslinker remains with the cellular component, not with the oligonucleotide. Since the photocrosslinking step can be performed quickly and efficiently at any desired time, one can obtain a 'snapshot' of oligonucleotide distribution and protein binding status in intact cells. Furthermore, the use of the cleavable crosslinker allows the target molecules to be labeled and analyzed by gel electrophoresis without interference from the oligonucleotide.

3.3. HPLC and other methods

Tidd et al. [76] evaluated the feasibility of modulation of N-ras oncogene in T15 cells using 9-mer methylphosphonate oligonucleotide analogues. Human colon carcinoma HT29 cells were used as an irrelevant cell line for determination of non-specific cytotoxicity and biological stability of the oligonucleotides. Biological stability of the analogues was obtained by purifying 100 μl of the culture fluids on C18-Sep-Pak cartridges (using 50% ethanol–water), and analyzed by HPLC on a Whatman-Partisil-10 ODS-3 column (C18 support). The methylphosphonates were found to be resistant to biochemical degradation and were

Table 2
Cell culture analysis of oligonucleotides

Method	Sample/substrate	Conclusion	Reference
Fluorescence			
Acridine	HL60	Cell uptake length and temperature dependent, faster for PO-oligonucleotides against PS-oligonucleotides	[30]
FITC	Human umbilical vein endothelial cells	Increased localization within nucleus with cationic lipid preparation	[53]
FITC, TRITC	Rat embryofibroblasts	Cellular distribution and degradation of oligo	[7]
Carboxyfluorescein (CF)	CCRF-CEM cells	100 fold increased efficiency with liposomes	[55]
FITC	Epithelial cell, primary human fibroblasts	Kinetics of oligonucleotide intake and accumulation	[56]
FITC	HL60, in RPMI 1640 medium	Length dependent oligonucleotide intake	[30]
Acridine	HL60, human Burkitt lymphoma, MOLT-4, DA1	Cellular accumulation temperature dependent	[57]
FITC	Murine spleen and lymph node cells	Heterogeneous and mitogen inducible oligonucleotide uptake	[58]
FITC	MOLT-4 cells	Uptake kinetics of chimeric oligonucleotides	[59]
CF	L929 cells	Internalization pathway and uptake kinetics	[60]
FITC	Hematopoietic cells (H9)	Better intake and residence of fluorescent oligonucleotides	[61]
Radiolabelling			
5′-^{3}P-	Human T-lymphocytes	Inhibition of *c-myc* expression	[63]
5′-^{32}P-	L929 mouse fibroblasts, Krebs 2 ascites carcinoma grown in mice	Degradation in 2–4 h within cells	[64]
^{3}H-	Mammalian cells	Upto 9-mer taken up intact	[65]
5′-^{32}P-	L929 cells	VSV-protein synthesis inhibition	[66]
5′-^{32}P-	Rouscher Red 5–1.5 cells	Mechanism of cellular uptake and internalization	[67]
^{35}S-	HL60	Oligonucleotide degradation kinetics	[68]
^{35}S-	K562/III	Time dependent cellular uptake, no saturation	[69]
^{35}S-	MCF-7WT, MCF-7ADR	PS-oligonucleotide accumulation 60% in cytoplasm and 5–6% in nucleus	[70]
5′-^{32}P-	CV-1 cells	Uptake PS-:MP-:PO-oligonucleotide 4:2:1	[49]
5′-^{32}P-	HeLa cells	Twice association of end labeled oligonucleotide than internally labeled	[71]
5′-^{32}P-	CCRF-CEM cells	Encapsulated oligonucleotides have 500 fold efficacy	[55]
5′-^{32}P-	PC-12 cells	7 h for oligonucleotide to enter nucleus	[72]
HPLC			
5′-^{32}P-	HeLa	Oligonucleotide degradation kinetics	[7]
Reverse phase	T15 and HT 29	MP-oligonucleotide showed biochemical stability and devoid of non-specific toxicity	[76]
Reverse phase	Xenopus oocytes	Covalently attached agents protects oligonucleotides from 3′ and 5′ exonucleases	[77]

devoid of any non-specific toxicity toward cultured human HT29 cells. Marianne et al. [77] stuided the stability of anti-messenger oligonucleotides inside Xenopus oocytes using modified (i.e. covalently attached intercalating agent at the 5′- and 3′-end of 8-mer, 9-mer or 17-mer oligonucleotides) and unmodified oligonucleotides, but end labeled either at 3′-end with [α^{32}P]ATP or at

5′-end with [γ^{32}P]ATP. The purified radiolabeled oligonucleotide was micro-injected into the cytoplasm of stage IV oocytes and incubated in the wells of micro-titration plates at 18°C for different times. The cells were lysed in homogenization buffer, centrifuged (10 000 × g, 5 min) and the supernatant was analyzed on HPLC. Covalently attached intercalating agents protected oligonucleotide degradation against 3′- and 5′-exonucleases, and the half life was increased to 40 min.

3.4. Summary of cell-culture studies

Cellular intake of oligonucleotides has been studied with various goals by different investigators with respect to internalization, organelle distribution, bio-stability, toxicity or anti-proliferative effects. Both fluorescence and radiolabeling approaches have been used. Flow cytometry has been mostly used for fluorescent labeled oligonucleotides. Limitations and difficulties in interpreting flow cytometric data were described earlier [61]. Quenching of the fluorescence within cells due to cellular components is a major problem. However, proper control and care must be exercised to circumvent this. Radiolabeling with different nucleides (especially ^{32}P and ^{35}S) have been extensively used. LSC alone or in conjunction with PAGE, autoradiography, and densitometry has been used for detection and quantification of such nucleides. LSC gives a total qualitative or quantitative measurement of oligonucleotide, unlike PAGE followed by densitometry which is characterized by both high sensitivity and resolution (Table 2y).

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