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Radiolabeled oligonucleotides for antisense imaging

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Abstract

Oligonucleotides radiolabeled with isotopes emitting γ -rays (for SPECT imaging) or positrons (for PET imaging) can be useful for targeting messenger RNA (mRNA) thereby serving as non-invasive imaging tools for detection of gene expression in vivo (antisense imaging). Radiolabeled oligonucleotides may also be used for monitoring their in vivo fate, thereby helping us better understand the barriers to its delivery for antisense targeting. These developments have led to a new area of molecular imaging and targeting, utilizing radiolabeled antisense oligonucleotides. However, the success of antisense imaging relies heavily on overcoming the barriers for its targeted delivery in vivo. Furthermore, the low ability of the radiolabeled antisense oligonucleotide to subsequently internalize into the cell and hybridize with its target mRNA poses additional challenges in realizing its potentials. This review covers the advances in the antisense imaging probe development for PET and SPECT, with an emphasis on radiolabeling strategies, stability, delivery and in vivo targeting.

Keywords

antisense imaging; antisense therapy; molecular imaging; mRNA; SPECT; PET; radiopharmaceuticals

1) Introduction

Oligonucleotides are the basic building blocks of nucleic acids (DNA and RNA), consisting of linear polymer chains of a few nucleotides, generally with molecular weight of 4000 to 10,000 Daltons. They carry vital biological information based on the nucleotide bases. Antisense oligonucleotides are unmodified or chemically modified single stranded oligomers engineered to contain a sequence complimentary to their target 'sense' nucleic acids, e.g. mRNA. The concept of antisense targeting is based on the astonishing specificity of complementary nucleic acid-base pairing mechanism elucidated by the rules of Watson-Crick and Hoogsteen[1, 2]. This mechanism has been well established in vivo, for treating diseases such as cancer employing the single-chain DNA–RNA oligonucleotides which are designed to interfere with the gene expression of target genes/mRNAs, namely antisense therapy [3, 4]. The same concept of complementary base-pairing mechanism of antisense oligonucleotides has been used as the basis to deliver radiolabeled oligonucleotides for (targeted) imaging of molecular events (such as gene expression), namely antisense imaging, in the target sites using non-invasive imaging modalities such as single photon emission

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computed tomography (SPECT) or positron emission tomography (PET). Radiolabeled oligonucleotides may also be used for monitoring their fate in vivo, thereby helping us better understand the barriers to its delivery. These developments have led to a new area of molecular imaging and targeting utilizing radiolabeled antisense oligonucleotides[5, 6].

The advancement in imaging technology has played a major role in evolution of antisense imaging[7]. Among the several technologies developed, the approaches using nuclear imaging, namely the SPECT and the PET are the most sought, because of their high sensitivity, selectivity and accessibility of instrumentation[8–10]. Also, the vast choice of radionuclides and versatility of conjugation chemistries available for radiolabeling antisense oligonucleotides are yet another key advantage with nuclear modality imaging[6, 11]. Furthermore, technological advances in the development of combined imaging utilizing hybrid SPECT-CT and PET-CT have gained high resolution and anatomical information obtained by the X-ray CT. Also, the breakthrough in preclinical small animal imaging systems such as the microSPECT-CT and microPET-CT have helped carry forward the advances made in translational research into clinical practice[8, 12].

Antisense imaging, has benefitted largely from the past and present breakthroughs achieved in antisense therapy, for e.g. in cancer treatment, primarily because many of their requirements had common goals[13]; for instance, both imaging and therapy require delivery of stable oligonucleotides which are resistant to degradation by enzymes such as nucleases and have low binding to plasma proteins in circulation[13]. Other features essential for antisense targeting is an ability of the radiolabeled antisense probes to overcome biological barriers such as evade the immune systems of the body, localize in the cells of interest and subsequently hybridize with the target mRNA[13]. Apart from these requirements the antisense imaging probe must remain sequestered in the target site for prolonged periods and at the same time be rapidly cleared from the non-target organs and tissues to obtain good contrast for imaging using SPECT or PET[13].

Although collective efforts in the imaging probe development and in vitro proof of concept antisense imaging studies are indicative of the positive strides made in the right direction, there still remain several challenges in realizing their in vivo potentials. While the synthesis of radiolabeled antisense oligonucleotide probes, which are stable in vivo remains a challenge, overcoming the biological barriers to their in vivo delivery is yet another predicament. Furthermore, the low ability of the radiolabeled antisense oligonucleotide to subsequently internalize into the cell and hybridize with its target mRNA poses additional challenges in realizing its potentials. There are several good reviews covering the overall development of antisense imaging [5, 13–16]. In this review we will narrow our focus to some of the advances in the antisense oligonucleotide imaging probe development for PET and SPECT imaging, with an emphasis on radiolabeling strategies, stability, delivery and in vivo targeting.

2) Probe development for antisense imaging

Single and double stranded DNA, RNA, and synthetic antisense oligonucleotides (Figure 1) have been labeled with a variety of radionuclides for in vitro evaluation[17]. During the early development of antisense hybridization technology, majority of the radiolabeling procedures involved the enzymatic incorporation of ^3H , ^{14}C , and ^{35}S labeled nucleotides into the DNA or RNA molecules[18, 19]. Although β -emitting ^{32}P or ^{35}S have been used for in vitro hybridization experiments, they are not useful for in vivo imaging.

For the purpose of SPECT or PET imaging, the strategy of radiolabeling the backbone of oligonucleotides has limited scope because oligonucleotide has only carbon, hydrogen oxygen, nitrogen and phosphorous as their backbone constituent elements (besides sulfur, in

the case of phosphorothioates). While absence of metal atoms in the oligonucleotide backbone (which may otherwise be replaced by their respective γ -emitting isotopes) impose some limitation for SPECT imaging, radiolabeling them with positron emitting isotopes such as ^{11}C , ^{15}O and ^{13}N having very short half life of 20.4 min, 2.04 min and 9.97 min respectively, are difficult to radiolabel and image in the required time frame for antisense imaging. Due to these shortfalls, antisense oligonucleotides are chemically modified at the 3' or 5' end to accommodate radiolabeled synthon addition[15, 20, 21] to achieve high yield, high specific activity and stability that are appropriate for antisense imaging with different isotopes possessing varied decay characteristics. Some of the commonly used isotopes for radiolabeling antisense oligonucleotides for SPECT and PET imaging are described below.

i) SPECT probes for antisense Imaging

One of the important requirement for the radioisotopes used in SPECT antisense imaging probe development is that the energy of their γ -emission and decay (half-life) should be favorable for radiolabeling and imaging within the required time frame of the study[22]. In this respect, $^{99\text{m}}\text{Tc}$ is a versatile radioisotope that emits readily detectable 140 KeV γ -rays with a half-life of ~ 6 h[23]. The time frame of its decay is ideally suited for labeling and imaging of antisense oligonucleotides for in vivo setting because it is long enough for scanning with SPECT instrument but at the same time keeps the radiation exposure low and helps reduce radiation burden to patients[23].

Another important prerequisite for successful antisense imaging probe development relies on synthesis of stable chelates, which refers to the formation of cyclic complex by co-ordination of a (radioactive) metal ion with a polydentate ligand [24]. The resulting radioisotope-chelate can be stably conjugated to antisense oligonucleotides[24]. Along these lines, there are several $^{99\text{m}}\text{Tc}$ -chelates that have been conjugated to antisense oligonucleotides. Dr. Hnatowich group has developed hydrazine nicotinamide- $^{99\text{m}}\text{Tc}$ chelates (SHNH) for labeling DNA[25]. For this purpose, after conjugation, the purified SHNH-DNA was radiolabeled with $^{99\text{m}}\text{Tc}$ by transchelation from glucoheptonate[25]. By this method a radiolabeling efficiency of $\sim 60\%$ was obtained[25]. In another study, Mardirossian et al. were successful in $^{99\text{m}}\text{Tc}$ labeling an amine derivatized 15-base peptide nucleic acid (PNA) oligomer through a modified mercaptoacetylglcylglycylglycine (MAG_3) chelator[26]. Labeling efficiencies up to 70% and specific activities as high as 0.4Ci/ μmol of PNA were achieved[26]. In yet another study in Hnatowich's group, $^{99\text{m}}\text{Tc}$ radiolabeled to an antisense phosphorothioate DNA was accomplished by three different methods using Diethylenetriamine- N,N,N',N',N'' -pentaacetic acid (DTPA), 6-Hydrazinopyridine-3-carboxylic acid (HYNIC) or MAG_3 [27] (Figure 2). The radiolabeling efficiency with all three chelators were found to be different, with the highest obtained for HYNIC-DNA ($60\% \pm 7.5\%$), followed by MAG_3 -DNA ($40\% \pm 5\%$), whereas DTPA-DNA had the lowest labeling efficiency of $<10\%$ [27]. Also, specific activities as high as 2.3Ci/ μmol were obtained for both HYNIC and MAG_3 -DNA whereas it was $<0.14\text{Ci}/\mu\text{mol}$ for DTPA-DNA[27]. Hjelstuen et al. used MAG_3 -tetrafluorothiophenol (TFTP) ester to form a conjugate with the 3' hexylamine derivative of a 20-base phosphodiester antisense oligodeoxynucleotide (ODN) in order to facilitate $^{99\text{m}}\text{Tc}$ labeling[28]. The complex had a radiochemical purity of more than 97% with specific activity of about 2.8Ci/ μmol [28]. Later on Hjelstuen and colleagues from the same group reported the development of the first kit for one-step labeling of oligonucleotides with $^{99\text{m}}\text{Tc}$ using *S*-benzoyl-mercaptoacetyldiglycine (MAG_2) [29]. In a study aimed at evaluating the antisense targeting properties of locked nucleic acid (LNA) a study with $^{99\text{m}}\text{Tc}$ labeled LNA was undertaken in Hnatowich's laboratory [30]. The radiolabeling of the LNA with $^{99\text{m}}\text{Tc}$ was achieved via MAG_3 and the radiochemical purity of the product ($^{99\text{m}}\text{Tc}$ -LNA) was $\sim 95\%$ after purification. In another recent study by Liu et al., a 18-mer antisense (AS) or sense

phosphorothioate-oligonucleotide (SON) targeting telomerase reverse transcriptase (hTERT) mRNA was radiolabeled with ^{99m}Tc using a bifunctional chelator namely, *N*-hydroxysuccinimidyl derivative of *S*-acetylmercaptoacetyltriglycine (*S*-acetyl NHS-MAG₃) [31]. They reported that labeling efficiencies of ^{99m}Tc -MAG₃-ASON/SON reached >70% within 15–30 min at room temperature with a specific activity of $\sim 0.25\text{Ci}/\mu\text{mol}$ [31]. The radiochemical purity after purification was also very high (>96%), indicating the significance of the modified chelator used in this study [31]. In yet another significant modification to standard methods, Liu et al. made several small but significant changes to the routine ^{99m}Tc radiolabeling procedures via NHS-MAG₃ chelator which resulted in high labeling efficiency (>90%) of a 15mer diethylenimine Oxide; tetrahydro-1,4-oxazine antisense oligonucleotide (MORF) with specific activity $>35\text{Ci}/\mu\text{mol}$, however the greatest advantage of this method was the complete elimination of purification procedures [32–34], that is generally expected for future antisense imaging kit development.

^{111}In is also an important radioisotope for antisense oligonucleotide probe development because it has a long physical half-life of about 2.8 days which is ideally suited for imaging as well as accessing the long term fate and biodistribution of oligonucleotides in vivo [35]. ^{111}In decays by electron capture and emit high-energy photons of 171 KeV and 245 KeV that can be used for imaging by SPECT.

A detailed study using ^{111}In -labeled oligonucleotide was performed by Dewanjee et al. [36], using DTPA-isothiocyanate conjugated phosphodiester and phosphorothioate oligonucleotide (ODN) that they synthesized earlier [17]. For this purpose, a 15-mer antisense oligonucleotide sequence complementary to c-myc mRNA was synthesized and aminolinked to form aminohexyloligonucleotide (AHON) [36]. The AHON was subsequently conjugated to diethylenetriamine pentaacetate (DTPA)-isothiocyanate (DTPA-ITC) to facilitate radiolabeling [36]. The incubation of $^{111}\text{InCl}_3$ with DTPA-ITC-AHON for 30 min gave a yield of 45%–60% and the optimum pH of chelation of DTPA-ITC-AHON probe with ^{111}In was found to be 6.5 [36]. Lewis et al. used ^{111}In to label a new antisense peptide-peptide nucleic acid (peptide-PNA) conjugate complementary to the first six codons of the bcl-2 gene [37]. For the conjugation, they used a new derivative of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), namely *N*-R-(9-Fluorenylmethoxycarbonyl)-*N*-ε-[tris(*tert*-butyl)DOTA]-L-lysine (Fmoc-K(DOTA)), that allowed ^{111}In labeled radio-chelates to be incorporated at any sequence position of the PNA conjugate [37]. Characterization of the conjugate (^{111}In -K(DOTA)-anti-*bcl*-2-PNA) by HPLC and ESI-MS indicated that the incorporation of ^{111}In was quantitative and the radiochemical purity was nearly 100% [37]. The specific activities were greater than $1\text{Ci}/\mu\text{mol}$, which were considerably higher than those reported previously by Dewanjee et al. [36]. Thus the Fmoc-K(DOTA) was found to be superior compared to DTPA for labeling with ^{111}In [37]. In another study Fujibayashi et al. synthesized a novel antisense probe utilizing a multi-aminolinked-oligonucleotide (ODN) conjugated to isothiocyanobenzyl-EDTA (IBE) to facilitate ^{111}In labeling. The use of multiple chelating sites (MCS) probe resulted in high ^{111}In labeling efficiency (>90%) and high specific activity of $1.29\text{Ci}/\mu\text{mol}$ [38]. Mamede et al. also used the same method described by Fujibayashi et al. for ^{111}In labeling of a 20-mer antisense oligonucleotides (oligo) and its biotinylated analog (oligo-bt) against c-erbB-2 oncoprotein, using 2-(p-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid chelate [39]. Labeling yields, determined by HPLC, were >85% for both the antisense oligomers and the specific activities of ^{111}In -oligo and ^{111}In -oligo-bt were 55–66 and 31–62 $\text{mCi}/\mu\text{mol}$, respectively [39]. In yet another study He et al., developed a method for incorporation ^{111}In into PNA oligomers [40]. For this purpose they used an amino acid (lysine) tagged mixed-base PNAs to facilitate DTPA coupling [40]. After HPLC purification they estimated that almost 80% of the PNA-DTPA was labeled

with ^{111}In [40]. In this regard it is important to note that radiolabeling of DTPA derivatized DNA and RNA analogs is easy and does not require post labeling purification [41, 42].

Isotopes of iodine have also been used for SPECT antisense imaging. ^{125}I Iodine with a physical half-life of ~60 days emits gamma radiation with maximum energy of 35keV. Another isotope of Iodine, ^{123}I Iodine has a comparatively shorter half-life of 13.22 hours and predominantly decays by emitting γ -radiation with energies of 159 KeV and 27 KeV. Both these isotopes of iodine were used as efficient gamma emitters for radiolabeling antisense oligonucleotides. Along these lines, Dewanjee et al. optimized the condition of radioiodination using a 25-mer actin mRNA probe modified by aminohexyl group at the 5'-terminal of the oligonucleotide and conjugated it with p-methoxyphenyl isothiocyanate (PMP-ITC) to facilitate ^{125}I labeling[17]. They found that ^{125}I pre-labeling of PMP-ITC (before conjugation to the oligonucleotide) was less convenient as opposed to ^{125}I radiolabeling of the PMP-ITC-conjugated oligonucleotide[17]. Also, they found that at higher stoichiometry of PMPITC/amino hexyl-oligonucleotide, the labeling efficiency increased with time of incubation, and reached yields up to 50–60%, whereas without PMPITC conjugation, only 4–6% of the ^{125}I was incorporated in the bases of the aminohexyl oligonucleotide[17]. Thus, the PMPITC conjugation in fact increased the specific activity of labeled oligonucleotide by a factor of 10 (80mCi/ μmol), compared to the one obtained by direct iodination (8mCi/ μmol)[17]. In another study, Panyutin et al. efficiently radiolabeled sequence-specific triplex-forming oligonucleotides (TFO) complementary to polypurine-polypyrimidine regions of human genes using ^{125}I isotope[43]. The specific activity of ^{125}I -TFO was ~40Ci/ μmol [44]. Dougan et al. successfully radioiodinated a modified stannyl oligodeoxyribonucleotide with ^{123}I which resulted in radiolabeling yields up to 97% with high specific activity of 15Ci/ μmol [45]. In another study Kuhnast et al. developed a more generalized and versatile approach to label antisense oligonucleotides with radiohalogens for imaging studies[46]. The method was based on the coupling of a 3' end phosphorothioate monoester oligonucleotide with a radiolabeled halogen-benzylacetamide compound (*N*-(4-[labeled]halogenobenzyl)-2-bromoacetamide) [46]. The coupling method was employed successfully to radiolabel oligonucleotides of varying lengths and with a variety of radiohalogens including ^{125}I [46].

ii) PET probes for antisense imaging

Positron emission tomography (PET) is considered as a more robust imaging technique for imaging molecular events compared to SPECT because it is at least ten times more sensitive than SPECT[5]. Further, positron-emitting isotopes can readily be substituted for naturally occurring atoms, producing fewer changes in the behavior of the radiolabeled molecules[5]. However as discussed earlier, the short half-life of PET isotopes necessitates its chemical conjugation (to the antisense oligonucleotide) and subsequent PET imaging to be done fairly quickly[5] which may restrict its application as an antisense imaging agent. This limitation has, at least in part, been overcome by utilization of quick radiolabeling and imaging which still makes their applications to antisense imaging feasible. Along these lines, ^{18}F Fluorine is a valuable isotope for antisense imaging utilizing PET because of its moderate half-life of ~110 min and high-energy positron emission (0.6335 MeV)[47, 48]. It should be noted that a method described above for labeling oligonucleotides for SPECT imaging with ^{125}I was initially successfully developed for PET imaging, using ^{18}F , by Kuhnast et al.[46, 49]. For synthesizing the ^{18}F probe, they used *N*-(4-[^{18}F]-fluorobenzyl)-2-bromoacetamide precursor to label the 3' end of a phosphorothioate monoester oligonucleotide [49]. Similarly de Vries et al. used the same PET precursor (*N*-(4-[^{18}F]-fluorobenzyl)-2-bromoacetamide) for reliably labeling an antisense oligonucleotide with good hybridization properties for inducible nitric oxide synthase (iNOS) mRNA [50]. Hedberg and Langstrom used 4-([^{18}F]-fluoromethyl)phenylbenzoate[51] or 4-([^{18}F]-fluoromethyl)phenyl isothiocyanate[52] for

labeling 5'-hexylamine modified oligonucleotides[52]. Pan et al. reported a modified oligonucleotide study in which the 5' conjugation of the radioactive probe was achieved by using a 5'-deoxy-5'-[^{18}F]-fluoro-O₄-methylthymidine[47]. The advantage of this method was that the entire reaction and workup could be completed within 4 h, a necessary criteria when working with short half-life radioisotopes such as ^{18}F [47]. Due to the increasing interest in the labeling of more stable peptide nucleic acids (PNAs) based probes, Tavitian's research group have developed a reliable method for the ^{18}F labeling of PNAs[53]. They demonstrated that it was possible to label PNAs in sufficient quantity and with high specific radioactivity of 1 Ci/mmol for their study[53]. The family of radionuclide probes with the above strategy developed by Tavitian's group covered a large panel of in vivo imaging techniques for both PET (positron emitters) and SPECT (γ -emitter) imaging[53].

Another PET isotope, ^{11}C Carbon is not very commonly used for antisense oligonucleotide imaging, primarily because of its very short half-life of ~20 min, which pose time constraints on the chemical synthesis, purification and imaging. However some researchers have developed novel strategies to overcome these limitations demonstrating in principle, the feasibility of ^{11}C labeling for antisense imaging. In one such study Kobori et al. used ^{11}C -labeling for real time imaging of mRNA expression[54]. They labeled a 5'-hexylamine derivative of phosphorothioate oligodinucleotide with [^{11}C]-ethylketene by taking advantage of the fact that [^{11}C]-ethylketene attacks -NH₂ residues much more readily than -NH and -OH residues[54]. The radiolabeled oligonucleotide (N-[1- ^{11}C]butyryl amino-hexyl oligodeoxynucleotide) was obtained with high specific activity of 5Ci/ μmol [54]. In another study Visser et al. were successful in labeling oligonucleotides with [^{11}C]-thymidine[55] indicating the feasibility of using ^{11}C as a PET isotope for antisense imaging.

The recent advances in $^{68}\text{Ge}/^{68}\text{Ga}$ generators design and its commercialization has revived the interest in ^{68}Ga radiolabeled PET agents[56]. ^{68}Ga is a valuable alternative to ^{18}F for PET imaging because it does not need an onsite cyclotron and also because of its high positron emission of 1.899 MeV [56, 57]. ^{68}Ga decays 89% through positron emission. Roivainen et al. used $^{68}\text{GaCl}_3$ for labeling antisense oligonucleotides targeting activated human K-ras oncogene using a macrocyclic chelating agent, 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA). The reaction of $^{68}\text{GaCl}_3$ with DOTA conjugated oligonucleotides was rapidly achieved within 10 min, at 100°C[58]. The radiochemical purity at 30 min was ~99% and the specific activity was about 68mCi/ μmol , and the radiolabeled oligonucleotide remained stable for >4h in water at room temperature[58]. In another study Lendvai et al. radiolabeled 17-mer antisense phosphodiester (PO), phosphorothioate (PS) and 2-O-methyl phosphodiester (OMe) oligonucleotides specific for point mutationally activated human K-ras oncogene using ^{68}Ga via a DOTA chelator for in vivo imaging with PET [59]. For the purpose of labeling they either used a non-concentrated or preconcentrated ^{68}Ga eluate [59]. The radiochemical purity of the antisense oligonucleotide tracers was >95% and no radioactive product was detected in the unconjugated macromolecules indicating that the label was attached to the chelator[59]. In yet another study, Lendvai et al. carried out the biodistribution studies of ^{68}Ga -labeled antisense for monitoring in vivo gene expression in rats[60]. For this purpose they labeled 20-mer DNA-LNA (locked nucleic acid) mixmer oligonucleotides specific for rat Chromogranin-A (Chg-A) mRNA using the same method as reported before[59]. The specific activity after radiolabeling was 219 mCi/ μmol [60].

3) Barriers to antisense imaging in vivo

While the development of radiolabeling strategies for antisense imaging probes is a great step forward in realizing the goals of radiolabeled oligonucleotides for antisense imaging, there are still several impediments to realize their potentials as an imaging agent in vivo,

including stability of the oligonucleotides, delivery to the cells of interest, transportation into the cells and targeting to mRNA as well as the concentration of target mRNA. All the factors listed above are inter-related to each other and affect antisense imaging in vivo. However the concept of antisense imaging, in principle, has been demonstrated on in vitro cell cultures and successfully shown in preliminary in vivo studies. These developments are encouraging for realizing our efforts to achieve the final goal of antisense imaging in vivo. This section will cover these barriers encountered in the antisense oligonucleotide probe development for in vivo imaging, and strategies to overcome these limitations.

i) Stability of antisense oligonucleotide probes in vivo

Although natural oligonucleotides have good hybridization potentials they exhibited variable stability resulting in vivo plasma half-life ranging from few seconds for oligoribonucleic acids (ORN) to few minutes for oligodinucleotides (ODN)[15]. In order to overcome this limitation chemical modification of the backbone was undertaken, while still trying to retain the original sequence as close as possible to their natural counterparts[61–63]. The chemical structures of DNA, RNA and some of their synthetic analogues are enlisted in Figure 1. The replacement of a non-bridging oxygen of the phosphate group in the natural oligonucleotides with a sulfur moiety resulted in the phosphorothioate backbone, which rendered higher resistant to nuclease degradation[64]. Unfortunately, this modification resulted in introduction of a chiral center on the phosphorous atom, which in turn resulted in cleavage/ degradation of some of the unfavorable configuration of the oligonucleotide by stereoselective enzymes such as 3'-exonucleases[65, 66]. Modified oligonucleotides such as methylphosphonates were found to be resistant to nucleases degradation but they had lower solubility in water (<1mg/ml)[67, 68]. Zhou et al. used a modified backbone containing both phosphorothioate and methylphosphonates, which resulted in maintaining their nuclease resistance yet improving solubility compared to methylphosphonate oligonucleotides[67]. In another study, modification of the 2' OH group of the ribose sugar of an oligoribonucleic acid to 2'-O methyl group resulted in high nuclease stability as well as remarkable hybridization ability[69]. Pandolfi et al. reported that the terminal end-capping of oligonucleotides with alkyl chains such as 1,3 propanediol or 1,6 hexane diol resulted in > 12 times stability to 3' and 5' exonucleases compared with the native oligonucleotides[70]. In another study, Agarwal et al. used novel synthetic strategies to arrive at mixed backbone oligonucleotides (MBOs) in order to increase in vivo stability[71]. They observed that some of the phosphorothioate oligodeoxynucleotides containing 2'-OMePO linkages remained stable toward endonucleases[71]. The other stable modification of the oligonucleotide backbone includes diethylenimine oxide tetrahydro-1,4-oxazine or morpholine oligonucleotides (MORF) [72, 73]. In experiments detailed by Hudziak et al., it was demonstrated that morpholino phosphorodiamidate oligomers were immune to a wide range of nucleases, including several DNases and RNases as well as degradative enzymes in serum and liver homogenate[72]. Apart from stability improvements, morpholino oligonucleotides also exhibited little or no non-antisense activity, afforded good water solubility, and had the advantage of being prepared at low production costs[72]. In an in vivo study conducted in Hnatowich's laboratory, ^{99m}Tc-labeled-phosphorodiamidate morpholinos (MORFs) showed high stability apart from good pharmacokinetics in vivo in mice[74]. In other noteworthy modification of oligonucleotides, the sugar-phosphate backbone was entirely substituted by a N-(2-aminoethyl)glycine, resulting in what is called a peptide nucleic acid (PNA)[1, 75–77]. Testing of the radiolabeled PNA on serum samples by size exclusion chromatography (SEC) and high-performance liquid chromatography (HPLC) analysis revealed that majority of the radioactivity were still associated with the PNA indicative of its prolonged plasma stability[26]. Intraperitoneal (i.p.) administration studies revealed that ^{99m}Tc-PNA displayed high stability, good complement targeting ability and favorable pharmacokinetic properties in vivo [26]. Among other modified oligonucleotides, those in which the ribose sugar moiety

is fixed either in the C3' or C2' conformation using one or more nucleotide building blocks is called a locked nucleic acid (LNA)[78]. Kurreck et al. designed antisense oligonucleotides containing locked nucleic acids (LNA) and compared them to 18 mer DNA oligonucleotides, phosphorothioates among others[79]. They found that the LNA oligonucleotide had 10-fold higher human serum stability compared to unmodified counterparts[79]. LNAs were also found to have unprecedented affinity and metabolic stability in vivo, attractive for application of antisense technology in vivo[78]. The above modifications of the oligonucleotide backbone indicates that chemical modification using novel methods indeed has a profound impact on in vivo stability which in turn results in improved pharmacokinetics and biodistribution properties of the antisense oligonucleotides.

ii) Antisense targeting of radiolabeled oligonucleotides

Although there are several radiolabeling probes and methods to label oligonucleotides for antisense imaging and monitoring gene expression in vivo, the successful application of antisense oligonucleotides necessitates its preliminary testing on cell cultures. The pioneering work by Urbain et al., revealed encouraging results of localization due to antisense mechanism based on scintigraphic imaging of oncogene with antisense probes[80]. Also, the work by Zhang et al. and Mardirosian et al. revealed several factors of chemical modification of oligonucleotides and their effect on in vitro cell accumulations [27, 81, 82], which may be translatable for in vivo antisense imaging. However, it should be noted that the in vitro results obtained from some of the antisense oligonucleotide studies do not always reflect their in vivo behavior. For instance, a study by Goodchild and coworkers revealed that an oligonucleotide labeled with [³²P] was completely degraded when injected intravenously into rabbits, whereas they found them to be stable when incubated in freshly drawn rabbit serum or human blood[83]. Although, the stability of antisense oligonucleotides have predominately indicated to depend on the sugar-phosphate backbone modifications as discussed before, the structural modification after chelation with different chelators and radiolabeling isotopes may impart additional complexity with regard to their in vivo stability. Similarly, while it is presumed that the in vitro results on stability or chemical modification of antisense oligonucleotides may be helpful in predicting their in vivo behavior, some studies have proven otherwise. For instance 3'-exonucleases in serum and cultured cells was found to be responsible for poor nucleases stability of 3'-exposed oligonucleotides in vitro[84–86] and capping that position (instead of the 5' position) by radiolabeling rendered higher stability towards nucleases degradation in vitro[87]. However the study by Zendegui et al. suggested that G-rich oligonucleotides minimally modified at the 3'-end, are relatively more stable in vivo and had distribution kinetics favorable for therapeutic use[88].

The pharmacokinetics and tissue distribution are also important parameters to determine the targeting functions of labeled antisense oligonucleotides for imaging applications in vivo. As discussed in prior sections, antisense oligonucleotides are prone to degradation due to presence of exonucleases and have poor stability in vivo, which further impose strict pharmacokinetic parameters to be met in order to observe desired antisense targeting effect. In this regard, monitoring the fate of radiolabeled antisense oligonucleotides in vivo utilizing imaging modalities such as PET and SPECT are invaluable for translation of these agent from lab to the clinic [89]. In order to assess the in vivo pharmacokinetics of any oligonucleotide probes, it is imperative to consider all the issues relating to its stability, specificity/targeting ability, cell internalization functions as well as solubility characteristics. With regard to in vivo clearance of antisense oligonucleotides derivatives, the evidence strongly suggests that phosphodiester oligonucleotides are rapidly cleared from blood within few minutes and radioactivity uptake are predominantly found in kidney and liver[90]; whereas phosphorothioate oligonucleotides, exhibited biphasic plasma clearance with a

primary short elimination of <1h followed by a long secondary clearance ranging between 1–2 days[90]. PNA derivatives also showed characteristics of rapid clearance through the kidneys and good plasma stability during circulation[26]. In another study with regard to the backbone structure and length of oligonucleotide, Langstrom et al. found that the in vivo biodistribution was influenced by the length of oligonucleotides [91].

Methylphosphonate oligonucleotide and methyl RNA are also attractive for imaging because they exhibited rapid body clearance predominantly excreted intact in the urine[68, 89]. In an effort to better understand the oligonucleotide radiotracer characteristics Tavitian et al. conducted studies in monkeys using 3'-end-¹⁸F labeled oligonucleotides [89]. Their main focus was to evaluate the difference between phosphodiester, phosphorothioate and 2'-O-methyl RNA analogues[89]. Their data corroborated the results that the ability of the antisense oligomer to hybridize its target sequence was not compromised by their radiolabeling method; however, the backbone of oligonucleotides indeed had variable pharmacokinetics and tissue distribution profiles[89]. They were also successful in measuring the concentration of ¹⁸F labeled metabolites in the plasma during the PET measurements, thus opening the way for a novel method to quantitatively evaluate radiotracer tissue levels which are highly relevant for future radiolabeled oligonucleotide antisense imaging probe development and drug development[89].

Also, the solubility characteristics of the antisense oligonucleotides impact the intra-cellular transport of these agents across the cell membranes[92, 93]. Usually since radiolabeled oligonucleotides have poor lipid solubility, they are unable to cross the cell membrane effectively[92, 93]. Furthermore, the complex 3D-structures formed by RNAs such as stem-loop or hairpin structures impose conformational restrictions to hybridization with the complementary strand in vivo[92, 93]. Stein has reported that, out of all the ONs that are delivered, only 6%–12% of oligonucleotides targeting an RNA sequence are able to form the duplex necessary for the antisense effect[92, 93].

Many of the antisense oligonucleotides such as the phosphorothioate oligonucleotides have been known to bind to plasma proteins, which in some cases resulted in deleterious effects [94–96]. Studies by Yakubov et al. and Geselowitz et al. have illustrated some of the different types of protein binding interactions[97, 98]. They found that when a photoactivatable crosslinking of phosphodiester oligonucleotide was incubated with cell cultures, 90% of the oligonucleotides was found to be bound to high molecular weight (75–79 kD) cell membrane proteins[97, 98]. In another study by de Vries et al., although an oligonucleotide against inducible nitric oxide synthase (iNOS) mRNA was reliably labeled with [¹⁸F]fluorobenzyl)-2-bromoacetamide, it however was found to have very high non-specific binding in the cell which hindered its specific hybridization with iNOS mRNA[50].

Another concern allayed by researchers is that the cellular concentration of the target mRNA may not be sufficiently high enough for imaging by antisense hybridization[99]. It is interesting to note that the although the concentration of viral-RNA in infected cells may be very high, but in general, the mRNA coding for a given protein is less abundant than the protein itself[99]. For instance, a typical mammalian cell contains only picogram levels of RNA[100]. Although these values work out to a modest three to five hundred thousand RNAs per cell, the amount of mRNA may account for only to a small fraction of the total RNA content in the cell. Nevertheless it is interesting to note that there may be as high as 11 thousand different species of mRNA in a cell depending on the type or function of each cell [100]. Another concern allayed by researchers for the feasibility of antisense imaging is due to the fact that some target mRNAs comprise as much as 3% of the mRNA pool whereas others account for < 0.01%[100]. Some researchers thus speculate if these numbers work in favor of antisense hybridization for imaging[99]. Along these lines, Tavitian found that in a

pool of about two thousand different species of mRNA present in a unit cell, only ~250 of them were the real targets for their antisense oligonucleotides[15].

In another interesting study, Kedzierski et al. found that the expression of tyrosine hydroxylase mRNA (mRNATH) in catecholaminergic cells varied with the location of those cells in the rat brain[101]. For instance, they found that there were about 1800 mRNATH molecules per cell in the adrenal medulla whereas there were a greater number (~ 4500) of mRNATH per cell in the hypothalamus of young adult rats[101]. Another interesting finding of their study was that amount of mRNA also varied with the age of male rats[101]. In this regard, they found that the amount of mRNA in the adrenal of ~2 year old animals were 25 times that in the adrenal of 4-day-old pups, indicating that expression of mRNATH does not remain constant with the progression of age of animals[101]. Irrespective of these shortcomings, William et al. measured the mRNA concentration encoding for various proteins of interest and found that their concentration was about 0.3–0.13nM[102]. Their studies indicated that concentration of a particular mRNA sequence may be in the range of 0.1 to 10nM[15].

In evaluating the number of antisense DNA accumulating per cell, Zhang et al. observed statistically higher accumulation of antisense DNA in cancer cells compared to control DNA[82]. From the information derived from in vitro cell culture experiments such as the number of cells incubated with the radiolabeled probe and its specific activity values, they calculated that the specific accumulation of the radiolabeled antisense DNA molecules in each cell were higher (by few orders of magnitude) when compared to the steady state target mRNA concentrations. The number of target mRNA was assumed to be in the range of ~1–1000 copies per cell. In another study corroborating the results obtained above, an eleven fold higher accumulation of ¹¹¹In-labeled PNAs was observed in Raji cells that overexpressed bcl-2 mRNA in comparison to their accumulation in U937 cells that do not express the bcl-2 target mRNA. However in this study a transduction peptide (PTD-4) was conjugated to the radiolabeled probe in order to facilitate its delivery[16]. Irrespective of this modification, the study does support the antisense effect of the radiolabeled probe (wherein the corresponding accumulation was found to be >2000 antisense molecules per cell)[16]. Along these lines, the practicability of antisense oligonucleotide for in vivo imaging was elegantly demonstrated by Dewanjee et al. using mouse xenograft models[17]. This result strengthen the proof of concept of antisense mechanism in vivo and the feasibility of radiolabeled antisense probes as targeting tools for in vivo imaging.

The research in Hnatowich's lab has in principle been able to address some of the critical issues with regard to the feasibility and mechanism of localization for antisense oligonucleotide imaging probes and their degree of accumulation in both cell cultures as well as in animal models[103–105]. For instance, in cell culture studies statistically significant increased accumulation of the radioactive antisense DNA probes in LS174T colon cancer cells[82], ACHN kidney cancer cells[82], and 231 breast cancer cells[106] were observed[82, 106]. They also evaluated the specific uptake by antisense mechanism, by performing a blocking control study [82]. For this purpose the ACHN cells were incubated with radiolabeled antisense DNA after prior incubation with increasing concentration of unlabeled antisense DNA (from 7–100nM) [82]. It was observed that the accumulation of radiolabeled antisense DNA decreased in dose dependent manner in comparison to a control study in which the accumulation of radiolabel sense DNA with increasing concentration of unlabeled sense DNA was assessed under the same conditions[82]. To further support the observed antisense mechanism, Nakamura et al. performed a study to evaluate the accumulation of the radiolabeled antisense DNA in KB-G2 multidrug resistant cells (MDR+) in comparison to KB-31 (MDR+) controls, both in cell culture experiments and in animal tumor models[104, 105]. They found convincing evidence for the antisense mechanism from

both studies[104, 105]. For instance, they observed high accumulation of the radiolabeled antisense DNA in KB-G2 multidrug resistant cells (MDR++) cells in comparison to KB-31 (MDR+) controls in animals bearing tumors[105]. From the above positive observations it can be concluded that antisense oligonucleotides does seem to have promising potentials for targeting in vivo imaging warranting further investigations.

With regard to feasibility of antisense oligonucleotides for imaging, the evaluation of kinetic parameters are also critical in assessing the hybridization potentials of antisense oligonucleotides in vivo[107], partly because successful imaging depends on the route of administration, its rate of clearance and ability to selectively accumulate in the target tissues. Along these lines, Nakamura et al. found that when a radiolabeled antisense DNA probe was injected intravenously in mouse bearing KB-G2 tumors (overexpressing the MDR1 mRNA), only about ~400 antisense DNA molecules accumulated specifically in the tumor, while this number increased dramatically to ~60000 when they injected the radiolabeled antisense DNA probe directly into the tumor (by intra-tumoral injection)[104]. They attributed this difference in number due to the lower dose of the radiolabeled probe reaching the target (tumor), when administered through the i.v. route in comparison to intra-tumoral injection[104]. In this regard, the structure of the target mRNA also play an important role in hybridization function, for instance, Freier et al. have indicated that hybridization for antisense oligonucleotides can range from a very short duration of 10^1 - $10^{-1} \text{ M}^{-1}\text{S}^{-1}$ for structurally constrained targets up to 10^6 - $10^7 \text{ M}^{-1}\text{S}^{-1}$ for unstructured targets[107].

As seen from the above findings, it can be concluded that antisense oligonucleotide imaging probe development necessitates novel strategies to overcome several barriers for its efficient in vivo delivery and targeting; not limited to its in vivo stability towards nucleases, non specific protein binding interactions and ability to localize and hybridize with the target mRNA, but also to overcome the inherent limitations such as low or unpredictable concentration of target mRNA within the cells. Further the ability of the radiolabeled antisense oligonucleotides to remain bound to the target sites (mRNAs) for the time frame of imaging and fast clearance from non-target organs and tissues are some of the other criteria's that has to be met in order for arriving at favorable antisense imaging outcomes.

iii) Delivery of antisense oligonucleotides in vivo imaging

Although antisense oligonucleotides may be invaluable agents for imaging applications, it is difficult to realize their full potential in the absence of a proper delivery system due to the barriers discussed above[108]. It has been observed that a major proportion of naked oligonucleotides (for e.g., phosphorothioates) interacts with a large number of cell surface proteins, and the fraction that internalize into the cells also ends up internalized into the lysosomal/endosomal compartments[92], rendering them unavailable for hybridization to the target mRNA in the cytoplasm or nucleus[109]. Also, numerous experiments have concluded that uptake of naked oligomers occurs due to active transport but internalization in many cell types occurs rather slowly and are also found to be temperature dependent[97, 110, 111]. It is believed that oligonucleotides enter cells via the active processes of adsorptive endocytosis and pinocytosis[112], the relative proportion dependent on oligomer concentration, cell type and its activation state[112]. For instance B-lymphocytes are known to be poor moderators of oligonucleotide internalization[112].

As seen in the earlier sections, although some of the in vivo stability and delivery issues can be addressed by chemically modifying the oligonucleotide backbone or preferential capping of the labile or degradable groups on them, there still remains several challenges for efficient targeting of the antisense oligonucleotides to the preferred sites and subsequent intra-cellular penetration into cells. In one strategy the oligonucleotides were coupled with cell penetrating/translocating peptides to facilitate their delivery[113]. For this purpose Pooga et

al. employed cellular transporter peptides such as transportan[113] to deliver the coupled PNA into dorsal horn cells, thereby exhibiting suppression of gene expression of functional melanin receptors in rats[114].

In another study, Rosi et al. developed gold nanoparticle-oligonucleotide complexes for facilitating intracellular gene regulation[115]. These gold nanoparticle-oligonucleotide complexes, apart from being less prone to nuclease degradation and non-toxic to the cells, also exhibited higher binding affinity for complementary nucleic acids compared with the unmodified oligonucleotides and demonstrated >99% cellular uptake in several cells including RAW 264.7 (macrophage), HeLa (cervical carcinoma), NIH-3T3 (fibroblast), and MDCK (kidney) cells[115]. In order to enhance the intracellular localization and/or cellular uptake of these versatile complexes, Patel et al. from the same group modified the gold nanoparticle-antisense oligonucleotides with synthetic peptides[116].

In many studies, oligonucleotides were protected against nucleases degradation and accomplished improved cellular uptake (and internalization) by encapsulating them into versatile carriers or complexation with cationic delivery systems [117]. Reports indicate that the anionic charge of the oligonucleotides facilitates its efficient complexation with cationic polymers such as polylysine[118, 119], polyethyleneimine [120, 121], dendrimers[122, 123] or lipids containing cationic components or vesicular nanocarrier systems/liposomes[117, 124–126]. In this respect high molecular weight vectors plays a key role in dictating the in vivo pharmacokinetics and delivery of antisense oligonucleotides [127]. Along these lines, encapsulation of antisense oligonucleotides in high molecular weight nanoparticles such as liposomes can be favorable because they can take advantage of the vascular abnormalities or the enhanced permeability and retention (EPR)-effect to selectively accumulate in certain disease sites such as tumors[128–131]. However the release of the oligonucleotides from the liposome may have to be addressed. Also, encapsulation of oligonucleotides into liposomes may decrease antisense oligonucleotide access to tissues or cells outside of the vascular system, thus restricting their use[132].

In one study, Zhang et al. encapsulated ^{99m}Tc -labeled antisense phosphorothioate DNA into cationic liposome carrier to evaluate their in vitro cellular uptake and efflux kinetic behaviors[106]. They observed 4–5 fold increased accumulation when the liposome carrier was employed in comparison to same dose of naked DNA[106]. Further, micro autoradiography and subcellular fractionation confirmed the internalization and increased cellular accumulation for both antisense and control DNAs with cationic liposomal carrier system[106]. Although use of carrier systems such as liposomes does seem to generally protect and help transfect antisense oligonucleotides, Conard et al. observed that the delivery of antisense oligonucleotides using the same cationic lipids can yield variable transfection efficiencies[133]. Their results indicated that there is yet no clear evidence to demonstrate if the same lipid reagent is best suited for different DNAs for a single cell line[133], or if they can be generally utilized. These findings, however indicates that for each particular antisense oligonucleotide delivery fulfillment there is a need for careful evaluation of the carrier lipid/liposome, polymeric or other vector based delivery systems. Proving this fact is yet another result from a study conducted by Conrad and colleagues, utilizing cationic lipids for transfection of oligonucleotide[133]. They found that there were marked difference in the cationic lipids that were best suited for phosphorothioate-modified oligonucleotide (ODN)s and ethoxy-modified ODN transfection in particular cell types[133]. From their study they suggest that the physical and chemical form of the DNA dictated the choice of the preferred lipid for specific cell types[133]. In another study conducted by Nestle et al., they investigated the influence of presence or absence of a cationic lipid for the uptake and selective inhibition of a keratinocyte intercellular adhesion molecule-1 (ICAM-1) by phosphorothioate antisense oligonucleotides. Surprisingly their findings suggested that

antisense oligonucleotides performed well even in the absence of cationic lipids indicating that these antisense oligonucleotides interact with keratinocytes differently than other cell types[134]. Also, in some cases although the cationic lipids may protect and facilitate the delivery of the antisense ON to their target cells, they may interfere or inhibit the antisense hybridization function[135]. In one study by Maus et al., cationic liposome formulation containing dioleoyloxypropyltrimethyl ammonium chloride/dioleoylphosphatidylethanolamine (DOTMA/DOPE) used as a carrier for antisense oligonucleotide delivery inhibited vascular cell adhesion molecule-1 gene expression in human endothelial cells[135]. Fillon and Philip reported that cationic liposomes should be used with caution to deliver gene or antisense oligonucleotides to mammalian cells[136]. Based on their findings, positively charged lipids showed in vitro cytotoxicity towards phagocytic cells[136]. Taken together these studies indicate that although delivery of antisense oligonucleotides utilizing vectors such as positively charged liposomes and polymers may be useful, there is a need for thorough evaluation of their in vitro and in vivo fate and effectiveness in serving their purpose. Nevertheless several favorable outcome of using delivery systems have given hope that they may be useful for future antisense oligonucleotide imaging. In one such study conducted in Tavitian's group the in vivo pharmacokinetics and bioavailability of HIV antisense oligonucleotide utilizing a anionic lipoplex carrier was found to be superior to either the free form the oligonucleotide or the one encapsulated using a cationic lipoplex carrier[137]. The whole body dynamic quantitative imaging of the radiolabeled antisense probes in baboons revealed that the anionic lipoplexes were the most suited with considerably enhanced bioavailability and accumulation in organs such as the lung, spleen and the brain[137].

Summing up, these results give us an indicator that the imaging of antisense oligonucleotide in vivo is challenging and may rely on several factors such as base sequence, radiolabeling method, chemical form of the oligomer, target cell/tissue and delivery vectors employed to overcome the delivery to its target intracellular mRNA. Depending upon the goals of the study a judicious choice of carriers and methods would be beneficial in realizing the full potential of antisense oligonucleotide targeting and imaging.

Future Directions

Several studies using antisense oligonucleotide probes revealed important criteria's to be met in order to realize their in vitro and in vivo antisense imaging potentials successfully. From the current studies thus far, it can be concluded that the antisense probes as imaging agents in vitro has been successfully met to a large extent. This has been made possible due to fulfillment of several criteria's such as ease of antisense oligonucleotide probe synthesis/chemical modification, addressing their stability issues, adequate targeting ability of the antisense oligonucleotides to their cellular localization and efficient hybridization to target mRNAs. However for the translation of the antisense imaging for in vivo imaging there still is a need to address many factors such as in vivo fate of the radiolabeled oligonucleotides, biological barriers to their delivery, stability of the oligonucleotides in vivo and retention of the radiolabel antisense probes by target cells, as well as the clearance from non-target organs and tissues. Apart from these factors, there are some inherent limitations for antisense imaging due to low concentration of target mRNA, which in many cases, still remains elusive. Nevertheless the exciting developments and breakthroughs achieved thus far in radiolabeled oligonucleotide based applications hold great promise for non-invasive antisense imaging. The wide range of available tools such as optical, PET, SPECT and MRI coupled with the vast choice of probes and the explosive development of nanoparticle-based drug delivery systems with potential for signal amplification lays the foundation for in vivo antisense imaging. The current developments are leading the way forward holding great promise in future for this burgeoning field.

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Figure 1.

Chemical structures of DNA, RNA and their synthetic analogues. Figure legends: 1) DNA; 2) Phosphorothioate DNA; 3) Phosphoramidate DNA; 4) Peptide nucleic acid (PNA); 5) RNA; 6) 2'-methoxyethyl RNA; 7) Diethylenimide oxide-tetrahydro-1,4-oxazine (morpholino) oligomer; 8) Locked nucleic acid (LNA).

Figure 2.
Chemical structure of bifunctional chelators used for radiolabeling ^{99m}Tc .