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Detection of cyclin D1 mRNA by hybridization sensitive NIC–oligonucleotide probe

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ABSTRACT

A large group of fluorescent hybridization probes, includes intercalating dyes for example thiazole orange (TO). Usually TO is coupled to nucleic acids post-synthetically which severely limits its use. Here, we have developed a phosphoramidite monomer, **10**, and prepared a 2′-OMe-RNA probe, labeled with 5-(*trans*-*N*-hexen-1-yl-)-TO-2′-deoxy-uridine nucleoside, dU^{TO}, (Nucleoside bearing an Inter-Calating moiety, NIC), for selective mRNA detection. We investigated a series of 15-mer 2′-OMe-RNA probes, targeting the cyclin D1 mRNA, containing one or several dU^{TO} at various positions. dU^{TO}-2′-OMe-RNA exhibited up to 7-fold enhancement of TO emission intensity upon hybridization with the complementary RNA versus that of the oligomer alone. This NIC-probe was applied for the specific detection of a very small amount of a breast cancer marker, cyclin D1 mRNA, in total RNA extract from cancerous cells (250 ng/μl). Furthermore, this NIC-probe was found to be superior to our related NIF (Nucleoside with Intrinsic Fluorescence)-probe which could detect cyclin D1 mRNA target only at high concentrations (1840 ng/μl). Additionally, dU^{T} can be used as a monomer in solid-phase oligonucleotide synthesis, thus avoiding the need for post-synthetic modification of oligonucleotide probes. Hence, we propose dU^{TO} oligonucleotides, as hybridization probes for the detection of specific RNA in homogeneous solutions and for the diagnosis of breast cancer.

1. Introduction

Homogeneous fluorescence-based assays for detection of nucleic acids,[1] are widely used in various studies in molecular biology and biochemistry, for example single-nucleotide polymorphism,[2] real-time PCR[3,4] and detection of cellular RNA.[5] Detection of nucleic acids is highly important for diagnostics of genetic and infectious diseases, discovery of gene-targeted drugs, and other biomedical studies. Fluorescent nucleobases provide interesting opportunities for sensing the target DNA or RNA in extract mixtures as well as in living cells.[6,7] To become an effective tool for hybridization analysis, the synthetic oligonucleotide (ONs) must produce measurable hybridization-induced differences in fluorescence intensity. Labeled-single-stranded oligonucleotides having such properties would provide the basis for development of hybridization assays, suitable for the determination of complementary DNA and RNA targets.

Probes modified with one type of fluorophore, were designed for homogeneous assays, in which hybridization with the target nucleic acids leads to increase or decrease of fluorescence.[8,9,5] Among these fluorophores organic dyes such as cyanines, fluorecseins and rhodamines were used for developing of these hybridization probes.[10,11]

Another approach for modulation of fluorescence upon hybridization involves an intercalative transduction agent in nucleic acid hybridization assays, such as thiazole orange (TO). TO is a chromophore composed of a benzothiazole derivative and a quinolinium ring linked via a monomethine bridge,[12] which has low fluorescence in the free form.[13] TO has been reported to provide dramatically enhanced fluorescence upon dsDNA binding, because of the interruption of the twisting motion around the central methane bridge (Φ changes from 0.0002 alone in aqueous solution to 0.1–0.4 with DNA).[13] Recently hybridization sensitive probes, labeled with TO, have been developed, including PNA FIT (forced intercalation) probes,[14] and ECHO probes that are, doubly TO labeled hybridization-sensitive DNA probes, controlled by exitonic interactions of the dyes.[15] Although TO labeled PNA probes showed increased fluorescence upon hybridization with complementary

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DNA or RNA they suffer from several disadvantages, such as their lack of duplex stability and their costly synthesis.[16]

Previously, we reported on duplex-DNA probes labeled with 5-(*p*-methoxy-cinnamyl)-uridine for the detection of cyclin D1 mRNA.[8] This NIF (Nucleoside with Intrinsic Fluorescence) probe exhibited high quantum yield (Φ 0.12) and maximum emission (478 nm) which is 170 nm red shifted as compared to uridine. The fluorescence of the NIF-uridine analogue was quenched when incorporated into an oligonucleotide and was quenched even more (9.5-fold) upon duplex formation. Hence, cyclin D1 derived ds-NIF probe was incubated with RNA total extracts of cancerous cells containing high levels of cyclin D1 mRNA. Consequently, fluorescence increased due to de-hybridization of the ds-NIF probe, followed by hybridization to target mRNA (cyclin D1), and release of the ss-NIF probe. This process led to a 3-fold increase in fluorescence within 90 min at room temperature.

Here we attempted to increase signal-background ratio upon hybridization by a different approach- applying fluorescent Nucleosides bearing an Inter-Calating moiety (NIC) probes. Hybridization of NIC probe to a cyclin D1-derived mRNA was compared to our previously developed ds-NIF probes.

Specifically, we describe the synthesis and photopysical properties of TO labeled 2′-OMe-RNA probes in which dU^{TO} monomer, **1**, replaces uridine (Fig. 1). We compared the fluorescence of singly or doubly labeled single strands to that of the corresponding duplexes with complementary DNA and RNA. In addition, we analyzed the fluorescent properties of mismatched duplexes in a dU^{TO} opposite position, to deduce base-paring specificity and to study the hybridization properties of monomer **1**. Finally, we demonstrated the use of dU^{TO}-labeled oligonucleotide probes for the detection of cyclin D1, a breast cancer mRNA marker, in total RNA extract from human cancerous cells.

2. Results

2.1. Design of dU^{TO}-labeled-oligonucleotide probes

Previously, we reported on promising dsDNA probes labeled with 5-(*p*-methoxy-cinnamyl)-uridine NIF-probe for the specific detection of cyclin D1 mRNA.[8] ds-NIF probes specifically identified the target mRNA, however the change of emission intensity relatively to background fluorescent intensity was insufficiently high. Therefore, here we implemented an alternative approach which may trigger a more significant change in the fluorescent intensity upon hybridization of the probe to target mRNA. For this purpose we designed a nucleoside bearing a chromophore moiety, serving as an intercalator (NIC probe). The fluorescence of the latter was expected to be activated upon hybridization to the target mRNA (Fig. 2). The same target mRNA, cyclin D1, was chosen here, for the sake of comparison of the efficiency of the current NIC method to that of the previous NIF method.

One of the important properties of TO, is that the quantum yield has been reported to increase upon intercalative binding to DNA,

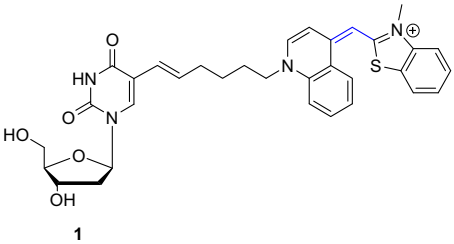

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Figure 1. TO-modified 2′-deoxy-uridine nucleotide, dU^{TO}.

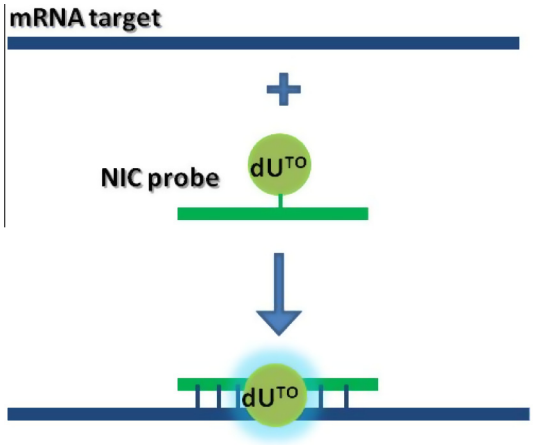


Figure 2. Schematic presentation of the principle of mRNA homogeneous detection using ssNIC-dUTO probe.

whereas the quantum yield of unbound dye is very weak.[13,17] In addition TO has relatively long absorption and emission wavelengths λ_{abs} 500 nm and λ_{em} = 530 nm, respectively. Therefore, we selected the TO dye as an intercalator of choice and conjugated it to uridine via a six carbons linker, at uridine C5 position to preserve the natural uridine H-bonding pattern. A six-carbon long hexenyl linker was selected to allow intercalation of TO, located at the end of the linker, between the stacked bases in the duplex of oligonucleotide probe-target.

Previously reported studies on PNA and DNA probes in which the carboxyalkyl linker was attached to the TO fluorophore, had shown the desired increase in the fluorescence upon hybridization (up to 30-fold).[18,16,19–21] However, in these studies a TO moiety was attached to the oligonucleotide post synthetically or as a base surrogate. Our goal here was to develop 2′-deoxy uridine conjugate, dU^{TO}, **1**, which can be incorporated anywhere in the sequence by automatic synthesis, and will result in the fluorescence enhancement upon intercalation of the moiety, following duplex formation with the complementary strand. We used single-stranded dU^{TO}-labeled 2′-OMe-RNA probes for the detection of cyclin D1 mRNA, a marker for breast cancer.

The cyclin D1 gene has been convincingly implicated in oncogenesis.[22–26] Cyclin D1 is a major player in the control of the cell cycle[27,28] and its expression is regulated at several levels, including mRNA transcription.[29] Among the dozen known human cyclin genes, only cyclin D1 has been implicated in oncogenesis[25] and its overexpression was shown to cause mammary cancer in transgenic mice,[30] and has been specifically implicated in breast cancer.[31] Furthermore, cyclin D1 overexpression has been observed in other types of cancer in which no amplifications or translocations occur.

We expected that our dU^{TO}-labeled probes would show enhancement of emission upon hybridization with target mRNA in total RNA extract, to allow the detection of cyclin D1 in cancerous cells that overexpress this biomarker. To test the applicability of dU^{TO}-labeled oligonucleotide probes for the detection of cyclin D1 in cancerous cell extracts, we selected a 15-mer sequence that targets nucleotides 644–658 of cyclin D1 mRNA. We chose a 15-mer long oligonucleotide since this is the minimal length that would ensure specific binding to target mRNA. The specificity of the sequence was assessed by BLAST (basic local alignment search tool). In addition to singly labeled oligonucleotides (ONs), several di-labeled ONs were prepared to examine the effect of more than one label on fluorescence of the single-strand and of the resulting duplex. We used 2′-OMe-RNA as a backbone of the dU^{TO}-labeled

oligonucleotides because 2′-OMe-RNA is known to exhibit a higher affinity for RNA targets than the corresponding DNA. In addition, 2′-OMe-RNA is chemically more stable than either DNA or RNA and is resistant to degradation by RNA- or DNA-specific nucleases.[32,33]

2.2. Synthesis of dU^{TO} phosporamidite, 9

The synthesis of dU^{TO} nucleoside, **1**, was based on a conventional dye synthetic protocol.[34–36] The thiazole orange moiety with an alkenyl linker was achieved by reaction of lepidine, **2**, under reflux in acetonitrile, for 4 days with *trans*-6-chloro-1-hexene-1-ylboronic acid pinacol ester **3**, to give compound **4** as a yellow solid in 85% yield (Scheme 1). Boronic acid pinacol ester, **4**, was coupled to DMT-protected 5-I-dU, **5**, under Suzuki conditions[37] using TPPTS, Na_2CO_3 and $Pd(OAc)_2$ in CH_3CN/H_2O (2:1), to obtain derivative **6** as a green solid in 88% yield. 2-Methylthio-*N*-methylbenzothiazolium iodide, **7**, was condensed with compound **6** in the presence of triethylamine in absolute ethanol to give the thiazole dye **8** in 83% yield as a red solid (Scheme 2).[38]

Compound **1**, was obtained in 80% yield upon treatment of **8** with 3% trichloroacetic acid in dichloromethane. Finally, dU^{TO} phosphoramidite monomer, **9**, required for oligonucleotide synthesis, was obtained in 78% yield, upon treatment of **8** with phophoramidite chloride and diisopropylethylamine. ^{31}P NMR spectrum showed two typical phosphoramidite signals at 148 ppm, for two diasterioisomers. The above monomer **9** allowed the facile incorporation of dU^{TO} into oligonucleotides at any internal or terminal position via a conventional oligonucleotide synthesis.

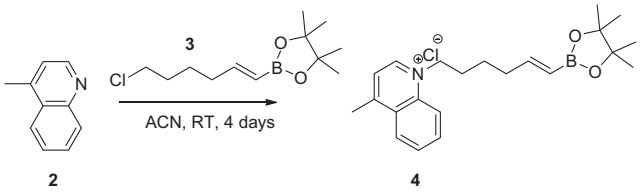
2.3. Synthesis of dU^{TO}-labeled oligonucleotide probes

Phosphoramidite **9** in dry dichloromethane was used in automated oligonucleotide synthesis, and the coupling time for the dU^{TO} monomer was prolonged to 10 min. Cleavage of dU^{TO}-labeled oligonucleotide from the solid support was achieved by employing fast deprotection, with 1:1 (v/v) 33% NH_4OH and 33% methylamine in ethanol at 65 °C, 10 min. All modified oligonucleotides were purified by HPLC and their identity was confirmed by MALDI-TOF mass spectroscopy.

First, to study the fluorescent properties of dU^{TO}-labeled oligonucleotide upon hybridization with target RNA, we synthesized as a model **dT7dU^{TO}dT7** capable of binding to the polyA tail of mRNA. Next, we synthesized four oligonucleotides, **ON1**-**4**, derived from cyclin D1 sequence and labeled with dU^{TO} at different positions: 4 or 10, di-labeled at positions 4 and 11; or 10 and 11 (Table 1).

2.4. Fluorescence properties of dU^{TO}-labeled oligonucleotide probes

Our main interest pertains to the detection of mRNA in compex environments, such as total RNA cell extract. Therefore, first we wanted to investigate the fluorescent properties of dU^{TO} in a nucleoside, an oligonucleotide, and upon binding to a specific target in homogeneous solution. dU^{TO}-modified nucleoside, **1**, showed



Scheme 1. Synthesis of reagent for the preparation of dU^{TO}.

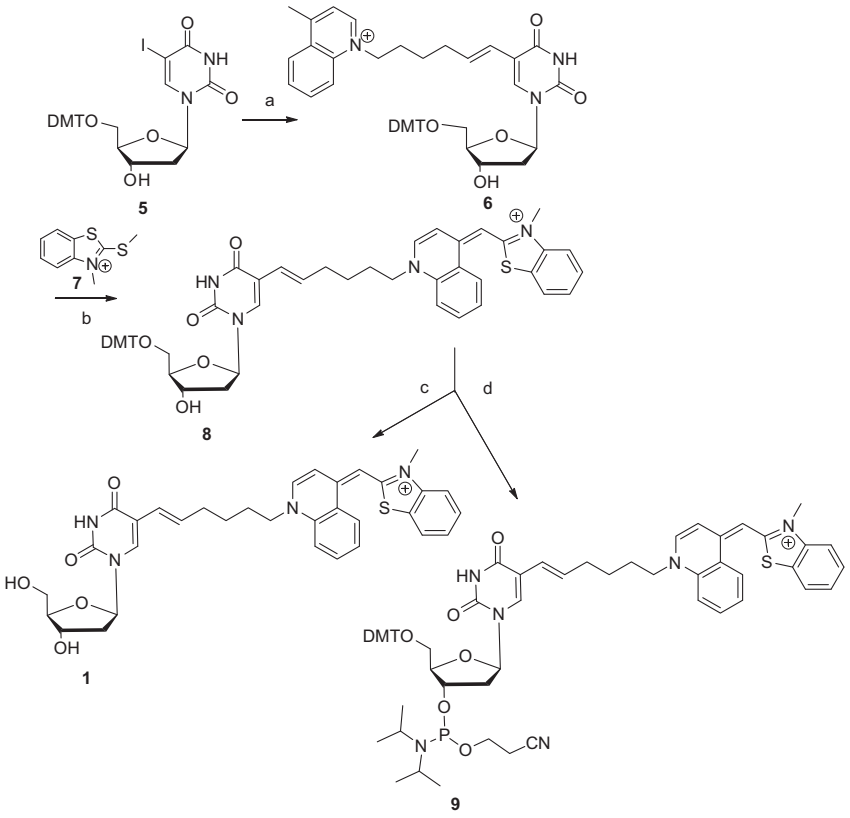
similar photophysical properties as TO (λ_{abs} 504 nm, λ_{em} 530 nm, Φ 0.0002 in PBS buffer). For this purpose we synthesized **dT7dU^{TO}dT7** oligonucleotide probe, as a model capable of binding to the polyA tail of mRNA. Specifically, we wanted to monitor any change in fluorescent emission of ss-dU^{TO}-oligonucleotide, upon selective hybridization to the complementary target RNA.

Emission spectra of **dT7dU^{TO}dT7**, showed weak fluorescence emission, upon excitation at 510 nm, at room temperature in PBS buffer. However, upon addition of complementary RNA, **A15,** a 19-fold increase in fluorescence was observed (Fig. 3). Next, we tested whether **dT7dU^{TO}dT7** probe can detect mRNA in heterogeneous RNA solution. The fluorescent emission was measured in the presence of mRNAs, containing complementary polyA tail, in total RNA cell extracts at a range of concentrations (30, 15 and 10 ng/μL). The mRNA extract was obtained from human U2OS osteosarcoma cell line. As a control, we used complementary cDNA, obtained from the mRNA total extract of the same cells, and random RNA strand 5′-GACGAUGGAGGGGCCGGACUCGUCAUACUC CUGCU-3′. The mRNA total cell extract was added after 3 min of excitation at 510 nm, to monitor if there are changes in fluorescent emission of the probe during the excitation. We measured the change in fluorescence emission at 535 nm, after addition of total RNA extract or cDNA, at room temperature, upon excitation at 510 nm (Fig. 4).

The probe became highly fluorescent almost immediately (30 s) after the addition of the total RNA extract, indicating hybridization with the polyA tails of mRNAs. Moreover, fluorescence is linearly dependent on the concentration of mRNA extracts. Upon addition of cDNA fluorescence intensity increased slightly, due to incomplete reverse transcriptase reaction and the presence of mRNA in cDNA. Likewise, we observed no increase of fluorescence intensity upon addition of a scrambled 30 nt RNA. We concluded, that the increase of the fluorescence intensity of dU^{TO}-labeled duplex occurred, due to the specific hybridization of dT7dU^{TO}dT probe to the target polyA.

Encouraged by these results we synthesized and studied the fluorescent properties of several single stranded, dU^{TO}-labeled, cyclin D1 derived oligo nucleotides **ON1**-**4** (Table 1). Corresponding duplexes were prepared for all oligomers. The fluorescence of probes **ON1**-**4** was measured before and after hybridization with target DNA and RNA. Table 2 summarizes absorption and emission maxima, quantum yields, and T_m values of **ON1**-**4** duplexes with a complementary strand. Measurements were performed that at 0.5 μM in PBS buffer (pH 7.4), at room temperature. The absorption bands of dU^{TO}-ONs were monitored at 483–512 nm. The emission spectra showed a single broad band at about 540 nm. **ON1**-**4** duplexes of with complementary RNA showed enhancement of fluorescence up to 6-fold versus that of **ssON1**-**4**. On the contrary, only a slight enhancement of TO emission was observed upon hybridization **ON1**-**4** with DNA as target.

The most significant $\Delta\Phi$ was observed for **ON2**-RNA duplex where quantum yield of dU^{TO} increased by 7-fold. However, almost no change of quantum yield was observed for **ON2**-DNA duplex (Fig. 5). The increase of fluorescence of dU^{TO} upon hybridization with complementary RNA, unlike the corresponding DNA, suggested that TO might intercalate with 2′-OMe-RNA-RNA hybrid in a different manner than that of 2′-OMe-RNA-DNA duplex. Generally, TO dye binds to the duplex through intercalation between base pairs of the duplex and/or major- or minor- groove binding.[39,40] To establish the mode of TO binding in our probes we measured CD spectra of **ON2** and the corresponding duplexes with DNA and RNA (Fig. 6). The single strand (**ON2**), and the duplexes showed negative and positive signals at 450–550 nm, which coincide with TO's absorption band. These signals might be due to different binding modes of the dye to the oligonuclotide.[41,42] Negative Cotton effect has been assigned to the intercalating mode of binding,



Scheme 2. Synthesis of dU^{TO} phosphoramidite, **9**[a]. [a]Reagents and conditions: (a) TPPTS, $Pd(OAc)_2$, Na_2CO_3 (3 equiv), CH_3CN/H_2O (2:1); (b) **7**, TEA, EtOH, reflux, 3 h; (c) 3% TCA in DCM, rt, 0.5 h; (d) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2, rt, 3 h.

Table 1
dU^{TO}-labeled cyclin D1-derived ONs

dU^{TO}-2′-OMe-RNA ONs	Sequence 5′ → 3′
Unmodified	CACUUGAGCUUGUUC
ON1	CACUUGAGC**dU^{TO}**UGUUC
ON2	CAC**dU^{TO}**UGAGCUGUUC
ON3	CAC**dU^{TO}**UGAGCUdU^{TO}GUUC
ON4	CACUUGAGC**dU^{TO}dU^{TO}**GUUC
Complementary	GAACAAGCTCAAGTG

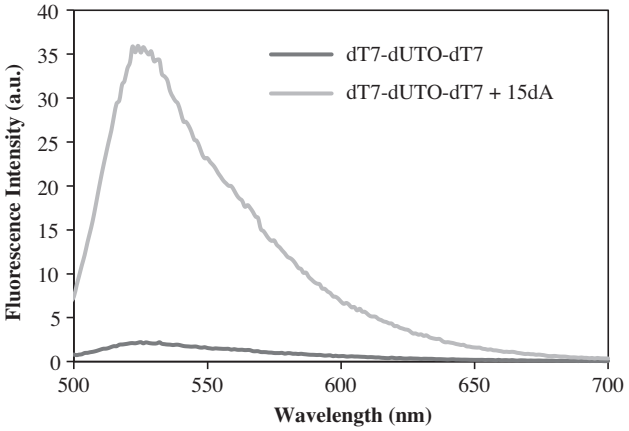


Figure 3. Emission spectra of dT7dU^{TO}dT7 probe alone and with complementary A15, (0.5 μM) measured in PBS buffer (pH 7.4, 25 °C), λ_{ex} 510 nm.

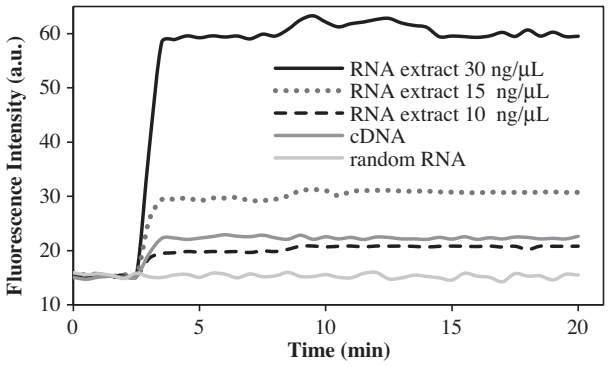


Figure 4. Measurements of time-dependent fluorescence of dT7dU^{TO}dT7 probe (0.5 μM) after adition of mRNA cell extract (30, 15 and 10 ng/μL), cDNA (30 ng/μL) and scrambled random RNA (5′-GACGAUGGAGGGGCCGGACUCGUCAUACUCCUGCU-3′) (0.25 μM), in PBS buffer (pH 7.4, 25 °C), λ_{ex} 510 nm, λ_{em} 535 nm. Total RNA extract was added at 3 min.

and it was assumed that the positive effect was caused by TO binding to the DNA/RNA groove.[43,44] The clear negative Cotton effect observed for **ON2**-RNA duplex indicates a predominant TO-binding through intercalation. In contrast, **ON2**-DNA hybrid showed a positive Cotton effect, indicating a less efficient intercalation and dye binding to the major or minor groove.[45,46]

The **ON1** exhibited only a modest enhancement in quantum yield upon hybridization with DNA and RNA, 1.6 and 2.7-fold, respectively. Likewise, only slight enhancements of quantum yields were observed for duplexes of dU^{TO} doubly-labeled oligonucleotides **ON3** and **ON4**, with either DNA or RNA.

Table 2
Photophysical properties of **ON1-4** and the corresponding duplexes with complementary DNA and RNA[a]

2′-OMeRNAs (**ONs**)		λ_{max} (nm)	Φ	λ_{em} (nm)	T_m (°C)
ON1	ss	511, 492	0.18	540	
	dsRNA-DNA	512, 488	0.30	540	49
	dsRNA-RNA	510, 488	0.41	537	55
ON2	ss	513, 490	0.04	533	
	dsRNA-DNA	512, 488	0.037	532	47
	dsRNA-RNA	510, 488	0.285	537	56
ON3	ss	510, 487	0.030	539	
	dsRNA-DNA	510, 488	0.046	540	48
	dsRNA-RNA	509, 481	0.051	541	57
ON4	ss	510, 486	0.018	539	
	dsRNA-DNA	510, 488	0.015	541	47
	dsRNA-RNA	508, 483	0.026	541	55
Unmodified duplex	dsRNA-DNA				46
	dsRNA-RNA				51

[a] All duplexes and single strand were measured at 0.5 μM, PBS buffer (pH 7.4, 25 °C), λ_{ex} = 490 nm. T_m values are given for all duplexes.

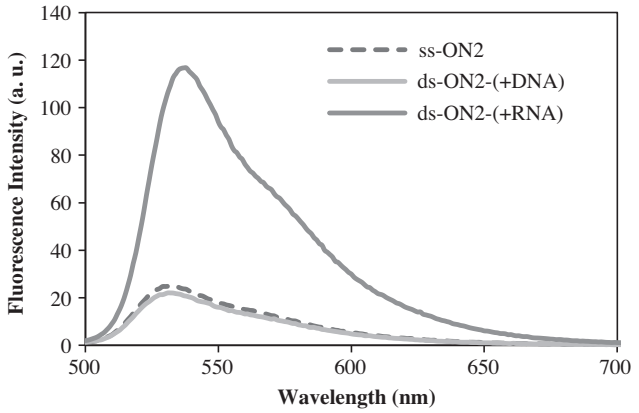


Figure 5. Emission spectra of ss-**ON2**, and ds-**ON2**/DNA(RNA) (0.5 μM) measured in PBS buffer (pH 7.4, 25 °C), λ_{ex} = 490 nm.

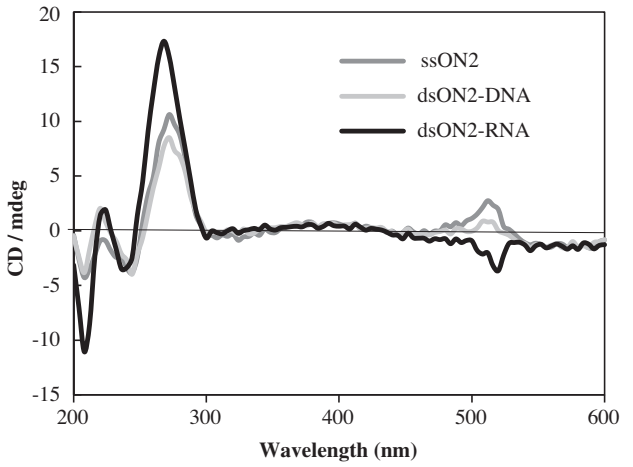


Figure 6. CD spectra of ss-**ON2**, and ds-**ON2**/DNA(RNA) (2.5 μM) were measured in PBS buffer (pH 7.4, 25 °C).

2.5. Effect of dU^{TO}-labeling on duplex stability

Development of hybridization sensitive fluorescent probe requires not only different potophysical properties in labeled-single strand versus labeled-duplex, but also duplex stability. Thus we assessed the effect of modified dU^{TO}-oligonucleotides on the stability of the duplexes (Table 2). T_m values of all dU^{TO}-ONs duplexes with complementary DNA and RNA strands were 47–49, and 55–57 °C, respectively. In most cyclin D1-derived labeled duplexes T_m increased by 1–6 °C as compared to the unmodified duplex for DNA and RNA duplexes (Table 2). There is the clear difference between duplexes in which the complementary strand was DNA (ΔT_m = +1–3 °C) or RNA (ΔT_m = 6–9 °C). These results indicate that the introduction of dU^{TO} to 2′-OMe-RNA strands stabilizes the corresponding DNA or RNA duplexes. We concluded that the thiazole orange moiety binds to the duplexes through intercalation and stabilizes the duplex structure by additional π-stacking interactions.

2.6. Effect of dU^{TO} labeling on matched versus mismatched duplexes

To test whether **ON1-4** can be used to sensitively detect sequence differences such as polymorphism, we investigated the changes of the fluorescence intensity of mismatched duplexes of **ON2**. When the base opposite to dU^{TO} in **ON2** was changed to a mismatched base (C/U/G), the quantum yields of the mismatched hybrids decreased by 14–27% versus those of the fully matched hybrid (Table 3). The small change in fluorescence suggests that a decrease in the RNA binding ability of dyes caused by a mismatched base pair is not significant (Fig. 7). Probably, the mismatched base pair partially destabilize the duplex structure, but does not affect the intercalation of TO dye to duplex binding site. It is noteworthy that the emission of the probes in the presence of mismatched RNA exhibits small change, indicating that the discrimination ability of dU^{TO} is limited.

2.7. Detection of cyclin D1 in RNA cell extracts by using dU^{TO}-labeled ON2

We applied **ON2** that exhibited the largest change of fluorescence intensity upon hybridization, to detect cyclin D1 mRNA in total RNA extracted from human U2OS ostreosarcoma cell line. Specifically, the fluorescence of **ON2** probe was measured, before and after addition of RNA cell extract from a cell line stably overexpressing cyclin D1 gene versus wild-type (WT) U2OS cells (Fig 7). Levels of cyclin D1 mRNA expression, were 3 times higher in U2OS cells overexpresing GFP-cyclin D1 than in WT U2OS cells.[8]

The fluorescence of **ON2** was measured in PBS buffer for 4 min and exhibited no change. After 4 min RNA extract containing high levels of cyclin D1 was added. As a controls we measured the fluorescence intensity of RNA extract alone (see Fig. S8, SI), and of extracts of cells that expressing basal level of cyclin D1. The emission intensity of dU^{TO} increased almost immediately (1 min) upon RNA extracts addition at room temperature (Fig. 8), indicating a rapid hybridization reaction between single stranded **ON2** probe and the target RNA. This experiment was performed in duplicates and on two different extract batches on three separate days using the same concentrations of ss-NIC probe and RNA extracts. The

Table 3
Hybridization of **ON2** with matched and mismatched ONs with and their photophysical properties[a]

	Sequence 5′ → 3′	T_m (°C)	Φ
ON2	CAC**dU^{TO}**UGAGCUGUUC		0.040
dsRNA-A	GAA CAA GCU CA**A** GUG	56	0.285
dsRNA-C	GAA CAA GCU CA**C** GUG	54	0.245
dsRNA-U	GAA CAA GCU CA**U** GUG	53	0.233
dsRNA-G	GAA CAA GCU CA**G** GUG	53	0.208

[a] All duplexes and single strand were measured at 0.5 μM, PBS buffer (pH 7.4, 25 °C), λ_{ex} = 490 nm. T_m values are given for all duplexes.

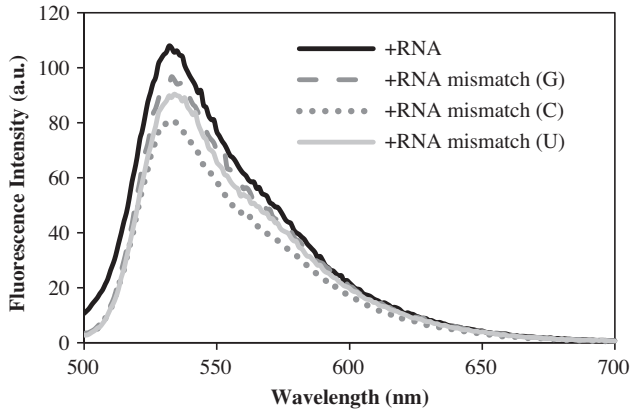


Figure 7. Emission spectra of **ON2** probe hybridized with RNA (match, mismatch-G, mismatch-C, mismatch-U), measured at 0.5 μM, λ_{ex} = 490 nm, in PBS buffer (pH 7.4, 25 °C).

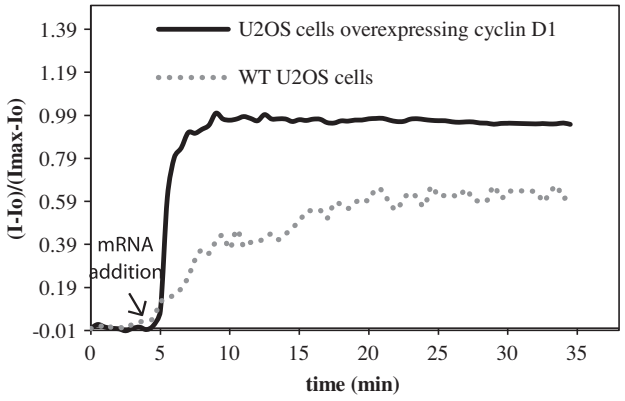


Figure 8. Representative measurements of time-dependent fluorescence of **ON2** after addition of mRNA extracted from cyclin D1 overexpressed and WT U2OS cells. Conditions: 0.5 μM probe in 80 μL PBS buffer (pH 7.4, 25 °C), and 250 ng/μL (after addition) total RNA extract, λ_{ex} = 510 nm, λ_{em} = 535 nm. RNA extract was added at 4 min.

fluorescent enhancement upon hybridization, ranged from 2.3 to 2.6-fold for RNA extracted from cyclin D1-over expressing cells as compared to that of WT cells. This is noteworthy considering that the overexpression of cyclin D1 is only 3 times higher in the overexpressing cells versus WT U2OS cells.[8] These results demonstrate qualitatively the efficiency of our dU^{TO} probe for the selective detection of a breast cancer marker, cyclin D1, in total RNA extracted from cancerous cells.

3. Discussion

Our main goal here was to improve the sensitivity of our previously published fluorescent NIF probes.[8] For this purpose we targeted the preparation NIC probes by incorporation of dU^{TO} monomer, **1**, into an oligonucleotide. We suggested that single stranded probe, labeled with TO intercalator, would increase fluorescence signal upon hybridization to the target, due to its affinity to dsDNA.[47] In duplex-NIF probes, the fluorophore is quenched upon duplex formation, and becomes fluorescent when released from the duplex by strand displacement process with the target. This process resulted in ca. 3-fold increase of fluorescence intensity. Here we targeted a better signal to noise ratio by using nucleoside-bound TO, known to increase emission by tens to thousands times upon ds-intercalation.

The novel NIC probes were based on dU^{TO}-labeled 2′-OMe-RNA probes hosting of the 2-deoxy-uridine bearing a TO moiety that was attached via six-carbon hexenyl linker at 5-position. The NIC probes were designed to be used for the detection of cyclin D1 mRNA, a breast cancer marker. We selected a 15-mer sequence from the cyclin D1 mRNA sequence, which is specific to this mRNA only and does not appear in any other endogenous mRNA. We incorporated dU^{TO} at various positions within the selected sequence and produced mono or doubly dU^{TO}-labeled oligonucleotides. Finally, we compared the ability of these dU^{TO}-labeled 2′-OMe labeled probes to that of ds-NIF probes,[8] to detect cyclin D1 mRNA.

Fluorescence intensities (which correlate with quantum yield) of the ONs and the corresponding duplexes were dependent on the location of the label in the ON. In particular, **ON2** in which the dU^{TO} was close to the end of the oligonucleotide exhibited low quantum yield in the single-strand (Φ 0.04) and high quantum yield in duplex with RNA (Φ 0.285). On the contrary, **ON1**, in which dU^{TO} was located in the middle of the oligonucleotide, exhibited similar high quantum yield in the single-strand and RNA duplex (Φ 0.18–0.41). No additive effect was observed for multi-labeled ONs. Moreover, di-labeled **ON3** and **ON4** exhibited very low quantum yields in duplexes with DNA and RNA (e.g., **ON3**, Φ 0.046, 0.051 and **ON3**, Φ 0.015, 0.026, respectively). This phenomenon may be explained by excitonic interactions between neighboring TO moieties.[18]

We studied the dependence of the thermo-stability of the labeled-2′-OMe-DNA and -RNA duplexes on the position of dU^{TO} label and number of labels. We found that incorporation of monomer **1** in ONs increased the stability of the resulting duplexes. In most labeled duplexes, T_m increased by 3–6 °C as compared to the unmodified duplex. The increased stability of these duplexes and their enhanced fluorescence intensities versus the corresponding ssONs, may indicate that the six-carbon hexenyl linker is long enough to lead to favourable stacking interactions through intercalation of the TO moiety.

Generally, the binding of TO to double stranded oligonucleotide involves several binding modes, the most important being intercalation and groove binding. The duplexes of **ON2** with RNA were more emissive and stable as compared to duplexes with DNA. The results of T_m and CD measurements suggested that the TO dye binds to RNA duplexes mainly through intercalation and thus thermally stabilizes the duplex structure.

These probes were designed to be used for the detection of specific mRNA. Therefore, after proving their ability to detect selectively polyA tails in total RNA cell extract by **dT7dU^{TO}dT7**, we demonstrated the ability of the optimal dU^{TO}-labeled probe, **ON2** to detect selectively cyclin D1 mRNA in an extract from cancerous U2OS cells. An immediate increase in fluorescence up to 2.5-fold was observed following the addition of the probe at room temperature to total RNA extract of cyclin D1 overexpressing U2OS cells versus WT U2OS RNA extract (both in concentration of 250 ng/μl) indicating the specific recognition of the cyclin D1 mRNA by the probe. It is noteworthy, that the level of cyclin D1 in overexpressing U2O2 cells is only ca. 3-fold higher than the basal level in WT cells.

The design of dU^{TO}-labeled ONs proposed here is different from recently reported singly-labeled probes.[14,15] Unlike the latter probes, here monomer **1** is used in conventional phosphoramidite solid phase oligonucleotide synthesis, without need for any additional post-synthetic modification of oligonucleotides. Furthermore, it can be substituted anywhere in the probe's sequence and in multiple positions. Substitution of the linker-TO moiety at C5- position of 2′-deoxy uridine, allows the preservation of the natural H-bonding pattern of U/T and does not hinder the duplex formation and stability. Furthermore the incorporation of dU^{TO} increased the stability of the corresponding RNA duplexes.

In contrast to NIF-probe, dU^{TO}-labeled NIC-probe, **ON2**, demonstrated remarkable ability to detect a small concentration of specific mRNA, cyclin D1 mRNA, in total RNA extract (250 ng/μL vs 1842 ng/μL, respectively). In addition, the maximum emission wavelength of NIC probe, is longer than that of NIF-probe, 540 nm versus 480 nm, thus allowing detection beyond the autofluorescence range of cells.

4. Conclusions

In conclusion, we have designed a series of fluorescent oligonucleotide probes based on the incorporation of dU^{TO}, uridine analogue **1**. The hybridization-sensitive, quencher free fluorescent NIC-probe, **ON2** proved to be applicable for the specific detection of a minute amount of a breast cancer marker, cyclin D1mRNA in total RNA cell extract (250 ng/μl) from cancerous U2OS cells. dU^{TO} monomer preserves the natural H-bonding pattern and can be used as a monomer in solid-phase oligonucleotide synthesis, without additional need for post-synthetic modification of ON probes. These results support the potential usefulness of **ON2** probe for the diagnosis of breast cancer.

5. Experimental section

Reagents and solvents were purchased from commercial sources and were used without further purification. All moisture sensitive reactions were carried out in flame-dried reaction flasks with rubber septa, and the reagents were introduced with a syringe. All reactants in moisture sensitive reactions were dried overnight in a vacuum oven. Progress of reactions was monitored by TLC on precoated Merck silica gel plates (60F-254). Visualization was accomplished by UV light. Medium pressure chromatography was carried out using automated flash purification system (Biotage SP1 separation system, Uppsala, Sweden). Compounds were characterized by nuclear magnetic resonance using Bruker AC-200, DPX-300 and DMX-600 spectrometers. ^{1}H NMR spectra were measured at 200, 300 and 600 MHz. Phosphoramidite monomer was characterized also by ^{31}P NMR in CD_3CN, using 85% aq H_3PO_4 as an external reference on Bruker AC-200, at 80 MHz. Chemical shifts are expressed in ppm, downfield from Me_4Si (TMS), used as internal standard. Compounds were analyzed under ESI (electron spray ionization) conditions on a Q-TOF micro-instrument (Waters, UK). Unmodified oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Modified oligonucleotides were synthesized by standard automated solid-phase method on an AKTA OligoPilot (GE healthcare) an ABI DNA/RNA synthesizer (Forster City, USA). MALDI-TOF mass spectra of oligonucleotides were measured with mass spectrometer in a negative ion mode with THAP matrix. Absorption spectra were measured on a UV-2401PC UV–vis recording spectrophotometer (Shimadzu, Kyoto, Japan). Emission spectra were measured using Cary Eclipse Fluorescence Spectrophotometer. Absorption and fluorescence spectra were recorded in PBS buffer containing NaC1 8.0 g, KC1 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, in water 100 mL (pH 7.4).

5.1. Synthesis of *trans*-4-methyl-*N*-(hexene-1-ylboronic acid pinacol ester-)-quinolinium chloride (4)

Lepidine (200 mg, 1.4 mmol), *trans*-6-chloro-1-hexene-1-ylboronic acid pinacol ester (684 mg, 2.8 mmol) and NaI (cat.) were dissolved in freshly distilled acetonitrile (10 mL). The mixture was heated under reflux for 4 days under nitrogen atmosphere. The solvent was evaporated and the residue was dissolved in a minimal amount of DCM and Et_2O was till the product precipitated. The product was filtered under vacuum, washed with Et_2O and dried under reduced pressure to give a yellow–green solid (500 g, 92%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.32 (d, 1H, J = 6 Hz), 8.35–8.34 (m, 1H), 8.26–8.25 (m, 1H), 8.18–8.15 (m, 1H), 7.98–7.96 (m, 2H), 6.55–6.52 (m, 1H), 5.41 (d, 1H, J = 18 Hz), 5.27 (t, 2H, J = 6 Hz), 3.00 (s, 3H), 2.26–2.24 (m, 2H), 2.10–2.07 (m, 2H), 1.69–1.66 (m, 2H), 1.24 (s, 12 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.8, 152.4, 149.3, 137.0, 135.2, 129.8, 129.4, 126.8, 123.4, 118.6, 83.1, 57.5, 34.9, 29.4, 24.9, 24.8, 24.7, 20.4 ppm. HRMS ES+ m/z: calcd for $C_{22}H_{31}BNO_2$ $[M]^+$ 352.245, found 352.244.

5.2. Synthesis of 5-(*trans*-4-methyl-*N*-(hexen-1-yl-)-quinolinium)-5′-DMT-uridine (6)

h Water/acetonitrile (8/4 mL) was added through a septum to a nitrogen–purged round bottom flask containing 5-I-5′-DMT-Uridine, **6** (1 g, 1.25 mmol), compound **3** (736 mg, 1.90 mmol), $Pd(OAc)_2$ (17.2 mg, 0.076 mmol), TPPTS (216 mg, 0.38 mmol) and Na_2CO_3 (106 mg, 4.56 mmol). The mixture was stirred under reflux for 3 h and monitored by TLC (8:2 DCM/MeOH). The solvents were evaporated. The deep green residue was separated on a silica gel column (5% MeOH in DCM). Product **7** was obtained as a green solid (870 mg, 88%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.92 (d, 1H, J = 6 Hz), 9.04 (s, 1H), 8.32 (d, 1H, J = 8 Hz), 8.26 (d, 1H, J = 6 Hz), 8.16 (t, 1H, J = 7.5 Hz), 7.94 (t, 1H, J = 7.5 Hz), 7.78 (d, 1H, J = 6 Hz), 7.74 (s, 1H), 7.40 (d, 2H, J = 7.5 Hz), 7.28–7.23 (m, 6 H), 7.17–7.16 (m, 1H), 6.79–6.78 (m, 4 H), 6.35–6.34 (m, 1H), 6.24–6.19 (m, 1H), 5.69 (d, 1H, J = 15 Hz), 5.07–5.05 (m, 2H), 4.71–4.72 (m, 1H), 4.05 (m, 1H), 3.73 (s, 6H), 3.42–3.41 (m, 1H), 3.33–3.32 (m, 1H), 2.92 (s, 3H), 2.46–2.39 (m, 2H), 1.94–1.92 (m, 4H), 1.50–1.42 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.3, 158.5, 158.0, 149.6, 148.6, 144.6, 136.9, 136.1, 135.7, 135.5, 131.5, 130.1, 129.9, 129.1, 128.0, 127.9, 127.8, 127.0, 126.8, 123.3, 121.6, 119.1, 113.2, 112.5, 86.6, 85.5, 84.7, 76.8, 63.3, 57.6, 55.3, 53.4, 40.7, 32.2, 29.2, 25.2, 24.4, 20.5 ppm. HRMS ES+ m/z: calcd for $C_{46}H_{48}N_3O_7$ $[M]^+$ 754.349, found 754.349.

5.3. Synthesis of 5-(*trans*-*N*-(hexen-1-yl-)-TO)-5′-DMT-uridine (8)

2-Methylthio-*N*-methylbenzo thiazolium iodide, **5**, (81 mg, 0.25 mmol) was added to compound **7** (200 mg, 0.25 mmol) in absolute ethanol (5 mL) and Et_3N (0.05 mL). The mixture was heated under reflux, under N_2 atmosphere. After 3 h, TLC analysis (CH_2Cl_2/CH_3OH 8:2) indicated the absence of starting material. The solvent was removed under reduced pressure. A red solid was obtained after purification on a silica gel chromotography (DCM/MeOH, 8:2), (156 mg, 66%). ^{1}H NMR (700 MHz, DMSO-d_6): δ 11.41 (s, 1H), 8.79–8.78 (m, 1H), 8.57 (d, 1H, J = 8 Hz), 8.08–8.06 (m, 1H), 8.05–8.03 (m, 1H), 7.98–7.96 (m, 1H), 7.79 (d, 1H, J = 8 Hz), 7.75–7.73 (m, 1H), 7.63–7.60 (m, 1H), 7.58 (s, 1H) 7.43–7.41 (m, 1H), 7.34–7.31 (m, 3H), 7.22–7.19 (m, 6H), 7.14–7.12 (m, 1H), 6.92(s, 1 H), 6.82–6.81 (m, 4 H), 6.21–6.17 (m, 2H), 5.61 (d, 1H, J = 16 Hz), 5.32 (d, 1H, J = 4 Hz), 4.54–4.51 (m, 2 H), 4.36–4.32 (m, 1H), 4.02 (s, 3H), 3.89–3.88 (m, 1H), 3.68 (s, 6H), 3.16–3.13 (m, 2H), 2.27–2.25 (m, 1H), 2.19–2.17 (m, 1H), 1.86–1.83 (m, 2H), 1.75–1.73 (m, 2H), 1.26–1.23 (m, 2H) ppm. ^{13}C NMR (175 MHz, DMSO-d_6): δ 162.1, 160.0, 158.1, 149.4, 144.2, 140.4, 136.9, 135.3, 130.2, 129.5, 127.7, 127.6, 126.7, 124.5, 124.2, 123.8, 122.8, 121.8, 113.1, 112.9, 111.0, 107.8, 88.1, 85.7, 85.6, 84.1, 70.3, 63.4, 54.9, 53.9, 39.6, 33.8, 32.4, 28.2, 25.5 ppm. HRMS ES+ m/z: calcd for $C_{54}H_{53}N_4O_7S$ $[M]^+$ 901.363, found 901.364.

5.4. Synthes of 5-(*trans*-*N*-(hexen-1-yl-)-TO)-uridine (1)

To a solution of **8** (50 mg, 0.05 mmol) in dichloromethane (1 mL) was added 3% trichloroacetic acid in dichloromethane

(2 mL) and the mixture was stirred for 30 min at room temperature. The resulting mixture was evaporated and purified by silica gel chromatography (DCM/MeOH, 7:3), to yield **9** as a red solid (37 mg, 80%). ^{1}H NMR (700 MHz, DMSO-d_6): δ 11.34 (s, 1H), 8.81–8.79 (m, 1H), 8.64 (d, 1H, J = 7.7 Hz), 8.14–8.13 (m, 1H), 8.06–8.05 (m, 1H), 7.99–7.95 (m, 1H), 7.96 (s, 1H) 7.80–7.79 (m, 1H), 7.76–7.73 (m, 1H), 7.63–7.60 (m, 1H), 7.44–7.42 (m, 1H), 7.39–7.38 (m, 1H), 6.40–6.37 (m, 1H), 6.16–6.14 (m, 1H), 6.07 (d, 1H, J = 16 Hz), 5.22 (d, 1H, J = 4 Hz), 5.06 (t, 1H, J = 4 Hz), 4.63–4.61 (m, 2 H), 4.25–4.23 (m, 1H), 4.02 (s, 3H), 3.79–3.77 (m, 1H), 3.62–3.55 (m, 2H), 2.16–2.08 (m, 4H), 1.90–1.86 (m, 2H), 1.50–1.48 (m, 2H) ppm. ^{13}C NMR (175 MHz, DMSO-d_6): δ 163.0, 162.0, 160.0, 159.9, 149.4, 148.4, 144.2, 140.4, 136.9, 136,2, 133.1, 131.5, 130.6, 129.7, 128.1, 126.7, 125.7, 124.4, 124.1, 123.7, 122.8, 121.9, 118.0, 112.9, 110.7, 107.8, 107.7, 88.1, 87.3, 84.1, 70.3, 61.0, 53.8, 45.6, 39.7, 33.7, 32.2, 28.2, 25.6 ppm. HRMS ES+ m/z: calcd for $C_{33}H_{35}N_4O_5S$ [M]$^+$ 599.232, found 599.229.

5.5. Synthesis of 5-(*trans*-*N*-(hexen-1-yl-)-TO)-3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite)-5′-DMT-uridine (9)

Compound **8** (200 mg, 0.16 mmol) was dissolved in CH_2Cl_2 (4 mL) in a flame dried two-neck flask under N_2. Diisopropylethylamine (0.48 mmol, 0.083 mL) and 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (54 μL, 0.2 mmol) were added to this solution. The clear yellow solution was stirred at rt for 19 h. The solvent was evaporated and the crude was immediately separated on silica gel using hexane/EtOAc (2:8) as an eluent containing 3% TEA. Compound **10** was obtained as a red solid (137 mg, 78%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.76–8.73 (m, 2H), 7.78–7.74 (m, 1H) 7.51–7.22 (m, 11H), 6.85–6.71 (m, 8H), 6.42–6.37 (m, 2H), 6.15–6.10 (m, 1H), 4.81–4.55(m, 1H), 5.58 (d, 1H, J = 16 Hz), 4.64–4.62 (m, 1H), 4.38–4.10 (m, 2H), 4.05 (s, 3H), 3.76 (s, 6H), 3.63–3.48 (m, 4H), 3.33–3.09 (m, 2H), 2.50–2.47 (m, 4H), 1.72–1.50 (m, 4H) 1.17–1.01 (m, 12H) ppm. ^{31}P NMR (80 MHz, CD_3CN): δ 148.61, 148.33 ppm. HRMS ES+ m/z: calcd for $C_{63}H_{70}N_6O_8PS$ [M]$^+$ 1101.471, found 1101.473.

5.6. Oligonucleotide synthesis, work-up and purification

The ON probes were assembled by DNA/RNA synthesizer applying phosphoamidite method. CPGs (3 μmol, pore size 500 Å) support and commercially available phosphoramidite nucleosides were used. The synthesized dU^{TO} modified nucleoside phophoramidite **9** was used at 0.1 M in dry DCM. The ONs were cleaved from the support with 1:1 (v/v) 33% NH_4OH and 33% methylamine in ethanol at 65 °C, for 10 min. The crude product was purified by HPLC on a C-18 column, and eluted with a linear gradient of 5–40% acetonitrile in 0.1 M TEAA (pH = 7), over 30 min at a flow rate of 3 mL/min. The oligomers were converted to the sodium salt using CM Sephadex C-25 equilibrated in NaCl and washed well with water. The identity of the ONs was determined by MALDI-TOF mass spectroscopy: **dT7dU^{TO}dT7** calcd for $C_{173}H_{203}N_{32}O_{103}P_{14}S$ ([M+Na])$^+$ 4864.75, found 4865.15 **ON1** calcd for $C_{178}H_{226}N_{51}O_{106}P_{14}S$ ([M+H])$^+$ 5238.92, found 5237.98; **ON2** calcd for $C_{178}H_{226}N_{51}O_{106}P_{14}S$ ([M+H])$^+$ 5238.92, found 5238.32; **ON3** calcd for $C_{201}H_{233}N_{53}O_{105}P_{14}S_2$ ([M+Na])$^+$ 5592.05, found 5592.67; **ON4** calcd for $C_{201}H_{233}N_{53}O_{105}P_{14}S_2$ ([M+Na])$^+$ 5592.05, found 5594.11. The yields of ONs were in the range of 95–98%.

5.7. Concentration and extinction coefficients calculations

Concentrations of oligonucleotides were calculated using the following extinction coefficients (OD260/μmol): The individual extinction coefficients used were ε_{dT} = 8400 M^{-1} cm^{-1}, ε_{dC} = 7050 M^{-1} cm^{-1}, ε_{dG} = 12,010 M^{-1} cm^{-1}, ε_{dA} = 15,200 M^{-1} cm^{-1}, $\varepsilon_{U\text{-}3'\text{-}OMe}$ = 10,000 M^{-1} cm^{-1}, $\varepsilon_{C\text{-}3'\text{-}OMe}$ = 9050 M^{-1} cm^{-1}, $\varepsilon_{G\text{-}3'\text{-}OMe}$ = 13,700 M^{-1} cm^{-1}, $\varepsilon_{A\text{-}3'\text{-}OMe}$ = 15,400 M^{-1} cm^{-1} and ε_{dU}^{TO} = 11,000 M^{-1} cm^{-1}. The extinction coefficients for the modified oligonucleotides were approximated by the linear combination of the extinction coefficients of the natural nucleotides and the extinction coefficient of the modified nucleoside. To account the base stacking interactions, this linear combination was multiplied by 0.9 to give the final extinction coefficients for the oligomers. The extinction coefficients of the duplexes (ε_D) are less than the sum of the extinction coefficients of their complementary strands (ε_{S1}, ε_{S2}), due to hyprochromic effect that should be taken into account.[48] Therefore, the extinction coefficients were calculated by the following equation:

$$\varepsilon_D = (1 - h_{260nm})(\varepsilon_{S1} + \varepsilon_{S2})$$

$$h_{260nm} = 0.287 f_{AT} + 0.059 f_{GC}$$

where f_{AT} and f_{GC} are the fractions of the AT and GC base pairs, respectively.

5.8. Hybridization of oligonucleotides

Solutions of labeled single-strands were mixed at room temperature with an equimolar amount of the complementary single strand oligonucleotides in PBS buffer (pH 7.4). Samples were hybridized by heating to 90 °C for 5 min and subsequently allowed to cool to room temperature over 2 h prior to measurements.

5.9. Absorption and fluorescence measurements

Absorption spectra of the probes were measured in PBS buffer containing 8.0 g NaC1, 0.2 g KC1, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, in water 100 mL (pH 7.4). The concentrations of the oligonucleotides were in the range of 2–2.5 μM. Absorbance was kept less than 0.05 AU in order to avoid inner filter distortion. Samples were measured in a 10 mm quartz cell. The fluorescence measurement conditions of oligonucleotides included 710 V sensitivity and a 5 nm slit. Samples were measured in PBS buffer (pH 7.4). λ_{ex} = 490 or 510 nm, λ_{em} = 500–700 nm range. The concentration of the samples was in the range of 0.4–0.5 μM. Samples were measured in a 10 mm quartz cell. The quantum yields (Φ) of the single-stranded and double-stranded oligonucleotides were calculated from the observed absorbance and the area of the fluorescence emission band. The fluorescence quantum yields of all oligonucleotides were determined relative to fluorescein in 0.1 M NaOH (λ_{ex} 494 nm, λ_{em} 521 nm, Φ 0.54) The quantum yield was calculated according to the following equation:

$$\Phi_F = \Phi_R I/I_R * OD_R/OD * \eta^2/\eta_R$$

Here, Φ and Φ_R are the fluorescence quantum yield of the sample and the reference, respectively, I and I_R are areas under the fluorescence spectra of the sample and of the reference, respectively, OD and OD_R are the absorption values of the sample and the reference at the excitation wavelength, and η and η_R are the refractive index for the respective solvents used for the sample and the reference.

5.10. CD measurements

CD spectra of fluorescent probes were measured in PBS buffer (pH 7.4) at 2.5 μM concentration, by using 1 cm quartz cell.

5.11. Thermal denaturation measurements (T_m)

The T_m values of duplexes were measured in. The absorbance of the samples was monitored at 260 nm and the temperature ranged from 20 to 85 °C with heating rate of 1 °C/min.

5.12. Cell culture and RNA cell extract

Human U2OS osteosarcoma cells were maintained in low glucose Dulbecco's Modified Eagle's Medium (DMEM, Biological Industries, Israel) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT). The clone overexpressing cyclin D1 contained a stable integration of a GFP-cyclin D1 plasmid under the control of the CMV promoter. RNA was extracted from wild-type U2OS cells and cyclin D1 overexpressing cells using TRI Reagent Sigma–Aldrich protocol. cDNA (1 μg RNA) was synthesized using the ReverseAid™ First Strand cDNA Synthesis Kit (Fermentas) with oligo-dT as a primer.

5.13. Fluorescence measurements of the ^{TO}U probe in total cell RNA extracts

Fluorescence measurements of **ON2** probe, in RNA extracts were performed at room temperature in PBS buffer (pH 7.4). The final concentration of the probe was 0.5 μM, while the concentration of the total RNA extracts was 250 ng/μL. The fluorescence was measured upon excitation at 510 nm. Samples were measured in a 10 mm quartz cell with a 1 cm path length. Duplicate samples were measured and the experiments were performed on three separate days using two different total RNA extract batches.

Supplementary data

Supplementary data (fluorescence and absorption spectra of dU^{TO} and **ON1**–**4**, selection of the probe's sequence**,** time dependent fluorescence spectra upon addition of **ON2** to total RNA cell extracts and NMR-spectra of compounds **1**–**9**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.03.033.

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