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Study of the base discrimination ability of DNA and 2′-*O*-methylated RNA oligomers containing 2-thiouracil bases towards complementary RNA or DNA strands and their application to single base mismatch detection

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Abstract—Precise detection of target DNA and RNA sequences using chemically modified oligonucleotides is of crucial importance in gene analysis and gene silence. The hybridisation and base discrimination abilities of oligonucleotides containing 2′-*O*-methyl-2-thiouridine (s^2Um) in homo- and hetero-duplexes composed of DNA and RNA strands have been studied in detail. When s^2Um was incorporated into RNA or DNA strands, the hybridisation and base discrimination abilities of the modified RNA or DNA oligomers towards the complementary RNA strands were superior to those of the corresponding unmodified oligomers. On the other hand, their base discrimination abilities towards complementary DNA strands were almost the same as those of the unmodified ones. The base discrimination abilities of 2-thiouracil base-containing oligonucleotide probes on slide glass plates were also studied. These modified probes exhibited efficient detection of mismatched base pairing.

1. Introduction

Through the high-throughput analysis of genome DNAs, it has become apparent that a large number of non-coding RNAs play an important role in the regulation of gene expression.[1–7] In particular, the analysis and utilisation of short double stranded RNAs such as short interfering RNA (siRNA) and micro-RNA (miRNA) have now become intriguing topics for medicinal and biological studies around the world.[1–3,5,8–10] The hybridisation-based methods used in high-throughput DNA chips/microarrays are favourable for analysing samples consisting of a large number of specimens.[11–13] However, in the case of short RNA or DNA targets, it is difficult to obtain precise and sufficient signal intensity because of their lower affinity to probes mounted on chips with inaccurate base recognition.[14–16] To overcome these problems in the detection of short targets, the utilisation of duplexes stabilised by modified nucleosides offers a powerful solution.[15,17–19]

It is known that 2-thiouridine (s^2U) favourably exists in the C3′-*endo* conformation.[20–22] Therefore, oligonucleotides containing s^2U derivatives form stable RNA duplexes with complementary RNAs.[23–27] Moreover, the T_m values of RNA duplexes containing an s^2U–G wobble base pair were considerably lower than those of RNA duplexes containing a matched s^2U–A base pair.[24,25] This fact indicated that s^2U forms a more stable base pair with A than G and exhibits the accurate base recognition. These properties of oligoribonucleotides containing s^2U or its derivatives are favourable for improving the detection of short target oligonucleotides. It seemed to us that RNA was not so stable and not suitable to carry out microarray experiments. It is well known that natural RNA is easily degraded by RNases. In our previous studies, T_m analysis of oligonucleotides containing 2′-*O*-methyl-2-thiouridine (s^2Um) and s^2U showed that these RNAs exhibited almost the same hybridisation and base discrimination abilities.[25] Therefore, in this study, we used 2′-*O*-methylated RNA as a stable RNA analogue. In this study, we report the hybridisation and base discrimination abilities of oli-

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gonucleotides containing s^2Um in various combinations of duplexes. RNA and DNA strands containing s^2Um were found to be superior to the unmodified strands with respect to their hybridisation and base discrimination abilities towards RNA strands. On the other hand, the base discrimination abilities of the modified RNA and DNA strands towards the complementary DNA strands were similar to those of the unmodified strands. Moreover, the hybridisation and base discrimination abilities of 2-thiouracil-containing probes mounted on glass plates were also studied, and these results revealed that the signal intensity and accuracy of the detection of short oligonucleotide targets were significantly improved.

2. Results

The hybridisation properties of the modified oligonucleotides towards their complementary strands were evaluated by determining their melting temperature (T_m). To compare the tendency of the hybridisation affinity and base discrimination ability of oligonucleotides containing s^2Um with the previous results, in this study, we selected similar sequences which were used in our previous studies. The base discrimination abilities were judged on the basis of the differences in the T_m values between matched and mismatched duplexes.

2.1. Hybridisation and base discrimination properties of DNA strands containing 2′-*O*-methyl-2-thiouridine (s^2Um) towards their complementary DNA and RNA strands

The T_m values of DNA strand containing s^2Um towards their complementary DNA and RNA strands were summarised in Figures 1A and 2A. In the case of the DNA strand containing s^2Um, as compared to the unmodified DNA strand, the s^2Um moiety did not enhance the hybridisation affinity towards a complementary DNA strand. However, the hybridisation affinity of a DNA strand containing s^2Um towards its complementary DNA strand was enhanced compared to that of a DNA strand incorporating 2′-*O*-methyl-uridine (Um) (The ΔT_m value observed between the Um–dA and s^2Um–dA base pairs was +2.6 °C). On the other hand, in the case of hybridisation with the complementary RNA strand, the hybridisation affinity of the DNA strand with s^2Um was almost equal to that of the unmodified DNA strand and superior to that of the DNA strand with Um. The base discrimination ability of the DNA strand containing s^2Um was judged on the basis of the differences in the T_m value between the matched and mismatched duplexes. These results are summarised in Figures 1B and 2B.

As far as the base discrimination ability of s^2Um is concerned, the s^2Um–G base pair was significantly destabilised compared to the Um–G and dT–G base pairs. In particular, the fidelity of a dT–G wobble base pair towards the complementary RNA strand was not sufficiently accurate (ΔT_m value observed between the dT–G and dT–A base pairs is only −4.0 °C). On the other hand, the T_m differences between the s^2Um–G and s^2Um–A base pairs towards their complementary DNA and RNA strands were over −10 °C in both the cases.

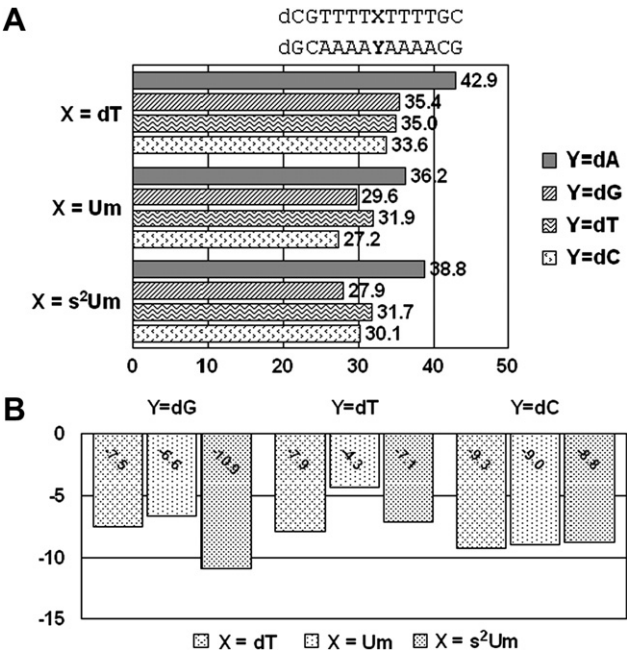

Figure 1. T_m analysis of DNA duplexes containing 2′-*O*-methyl-2-thiouridine (s^2Um). (A) Melting temperatures of oligodeoxyribonucleotides containing s^2Um, Um, or dT and their complementary DNA having fully matched or single base mismatched site at counterpart of modified base. Duplex sequences are shown above the chart. Melting temperature experiments were performed under following conditions: sodium phosphate buffer at pH 7.0 containing 150 mM NaCl. (B) Base discrimination of dT, Um or s^2Um in DNA duplexes. Values determined from T_m difference of fully matched and single base mismatched DNA/DNA duplexes.

2.2. Hybridisation and base discrimination properties of 2′-*O*-methylated-RNA containing s^2Um towards their complementary RNA and DNA strands

The T_m values and base discrimination abilities of 2′-*O*-methylated-RNA strands containing s^2Um towards their complementary DNA and RNA strands are summarised in Figures 3 and 4. In the case of s^2Um incorporated in 2′-*O*-methylated-RNA, the hybridisation affinities of the modified RNA oligomer towards complementary DNA and RNA strands were enhanced. In the case of s^2Um incorporated in 2′-*O*-methylated-RNA, the hybridisation affinities of the modified RNA oligomer towards the complementary DNA and RNA strands were enhanced. In the 2′-*O*-methylated-RNA/DNA duplex, the ΔT_m value observed between the Um–dA and s^2Um–dA base pairs was +7.4 °C, and in the 2′-*O*-methylated-RNA/RNA duplex, the ΔT_m value observed between the Um–A and s^2Um–A base pairs was +3.2 °C. These results well agree with those reported previously.[25] Moreover, the base discrimination ability towards the guanine base was also enhanced. The ΔT_m values were −7.2 and −4.9 °C for the 2′-*O*-methylated-RNA/DNA duplex and the 2′-*O*-methyl-

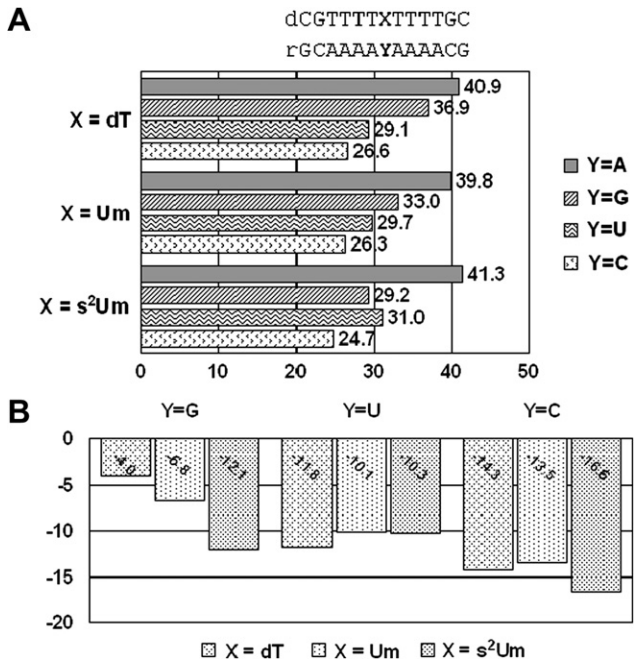

Figure 2. T_m analysis of DNA/RNA duplexes containing 2′-*O*-methyl-2-thiouridine (s^2Um). (A) Melting temperatures of duplexes formed between oligodeoxyribonucleotides containing s^2Um, Um and dT and their complementary RNA strands having fully matched or single mismatched site at counterpart of modified base. The sequences of the duplexes are shown above the chart. Melting temperature experiments were performed under following conditions: Sodium phosphate buffer at pH 7.0 containing 150 mM NaCl. (B) Base discrimination of dT, Um or s^2Um in DNA/RNA duplexes. Values determined from T_m differences of fully matched and single base mismatched DNA/RNA duplexes.

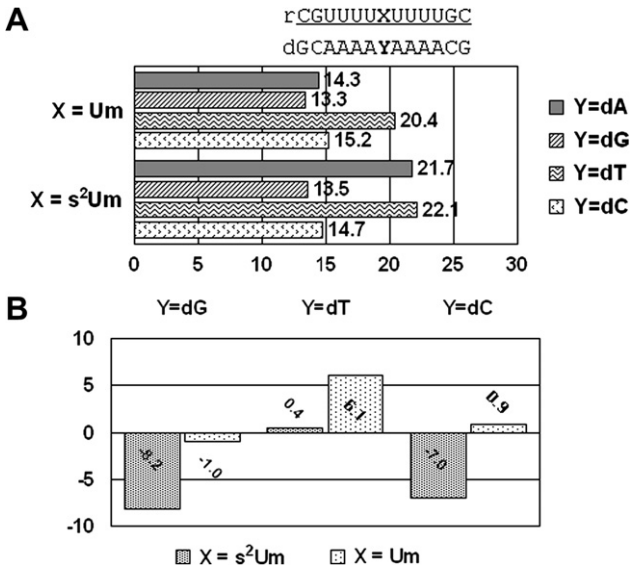

Figure 3. T_m analysis of 2′-*O*-methylated RNA/DNA duplexes containing 2′-*O*-methyl-2-thiouridine (s^2Um). (A) Melting temperatures of duplexes formed between 2′-*O*-methylated oligoribonucleotides containing s^2Um or Um and their complementary DNA strand having fully matched or single mismatched site at counterpart of modified base. Duplex sequences are shown above the chart. Underlined sequences are 2′-*O*-methylated oligoribonucleotides. Melting temperature experiments were performed under following conditions: sodium phosphate buffer at pH 7.0 containing 150 mM NaCl. (B) Base discrimination of Um or s^2Um in 2′-*O*-methylated RNA/DNA duplexes. Values determined from T_m differences of fully matched and single base mismatched 2′-*O*-methylated RNA/DNA duplexes.

ated-RNA/RNA duplex, respectively. Interestingly, in hybridisation with the complementary DNA strand, the accurate base discrimination ability of the 2′-*O*-methylated RNA with s^2Um was lost. Surprisingly, it was found that the Um–T and s^2Um–T mismatched base pairs were greatly stabilised (ΔT_m value observed between match and mismatch base pairs is +6.1 °C and +0.9 °C, respectively).

2.3. Effects of salt concentration on hybridisation and base discrimination abilities

The thermal stability and base discrimination ability of 2′-*O*-methylated-RNA/DNA duplexes were extremely different from those of the other duplexes, for example, the T_m values of their duplexes were observed at around 10–20 °C. This phenomenon was caused due to the stability of the rUU/AA region of the sequence used in this study. Sugimoto et al. reported that the nearest neighbour parameter of RNA/DNA duplexes, ΔG of rUU/dAA was only −0.2 kcal/mol.[28] This weak stabilisation effect induced a decrease in the stability of the duplexes. For increasing the stability of the 2′-*O*-methylated-RNA/DNA duplexes, we performed melting temperature experiments in high salt concentration conditions (10 mM sodium phosphate buffer at pH 7.0, 1 M NaCl). These results are shown in Figure 5. In the case of 1 M NaCl conditions, the T_m values of 2′-*O*-methylated-

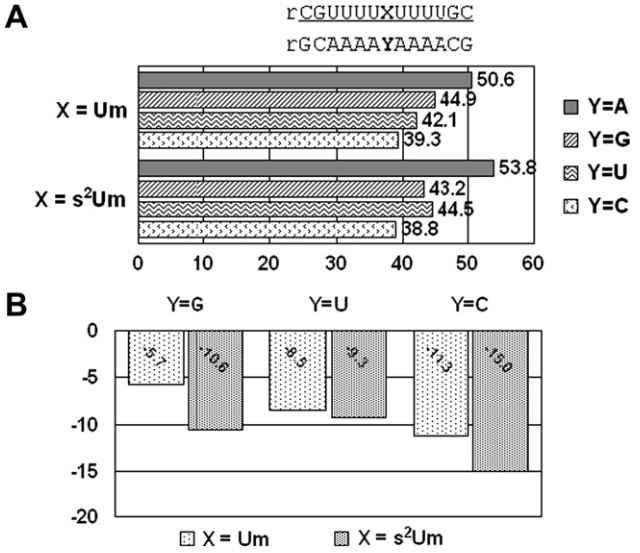

Figure 4. T_m analysis of 2′-*O*-methylated RNA/RNA duplexes containing 2′-*O*-methyl-2-thiouridine (s^2Um). (A) Melting temperatures of 2′-*O*-methylated oligoribonucleotides containing s^2Um or Um and their complementary RNA strand having fully matched or single mismatched site at counterpart of modified base. Duplex sequences are shown above the chart. Underlined sequences are 2′-*O*-methylated oligoribonucleotides. Melting temperature experiments were performed under following conditions: Sodium phosphate buffer at pH 7.0 containing 150 mM NaCl). (B) Base discrimination of Um and s^2Um in RNA duplexes. Values determined from T_m difference of fully matched and single mismatched 2′-*O*-methylated RNA/RNA duplexes.

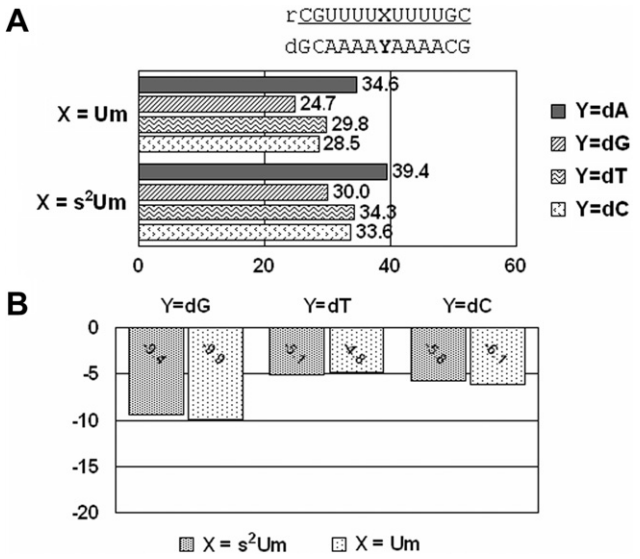

Figure 5. T_m analysis of 2′-*O*-methylated RNA/DNA duplexes containing 2′-*O*-methyl-2-thiouridine (s^2Um) in the presence of 1 M NaCl containing phosphate buffer. (A) Melting temperatures of duplexes formed 2′-*O*-methylated oligoribonucleotides containing s^2Um or Um and their complementary DNA strands having fully matched or single mismatched site at counterpart of modified base. Duplex sequences are shown above the chart. Underlined sequences are 2′-*O*-methylated oligoribonucleotide. Melting temperature experiments were performed under following conditions: Sodium phosphate buffer at pH 7.0 containing 1 M NaCl). (B) Base discrimination of Um or s^2Um in 2′-*O*-methylated RNA/DNA duplexes. Values determined from T_m differences of fully matched and single mismatched 2′-*O*-methylated RNA/DNA duplexes.

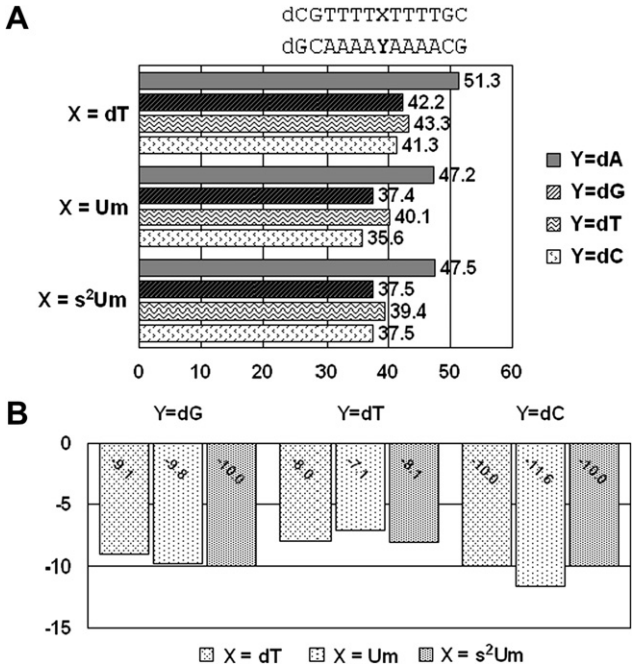

Figure 6. T_m analysis of DNA/DNA duplexes containing 2′-*O*-methyl-2-thiouridine (s^2Um) in the presence of 1 M NaCl containing phosphate buffer. (A) Melting temperatures of duplexes formed between oligodeoxyribonucleotides containing s^2Um or Um and their complementary DNA strands having fully matched or single mismatch site at counterpart of modified base. Duplex sequences are shown above the chart. Melting temperature experiments were performed under following conditions: Sodium phosphate buffer at pH 7.0 containing 1 M NaCl). (B) Base discrimination of dT, Um or s^2Um in DNA duplexes. Values determined from T_m differences of fully matched and single mismatched DNA/DNA duplexes.

RNA/DNA duplexes were significantly increased and the unusual stabilisation of the Um–T mismatch disappeared (Fig. 5A). However, the difference in the base discrimination ability between s^2Um and Um was almost the same (Fig. 5B). Similarly, for the DNA/DNA duplexes in 1 M NaCl conditions, the difference in the base discrimination ability between dT or Um and s^2Um was almost identical (Fig. 6B). On the other hand, the DNA/RNA duplexes in 1 M NaCl, the difference in the base discrimination ability between dT or Um and s^2Um was significantly different (Fig. 7B). Namely, s^2Um exhibited a large difference in wobble base pair discrimination (the ΔT_m value observed between s^2Um–A and s^2Um–G was −12.8 °C), though the use of dT and Um gave almost identical results (ΔT_m values are −6.2 °C and −7.5 °C, respectively).

2.4. Hybridisation and base discrimination abilities of 2′-*O*-methylated-RNA probe containing s^2Um on slide glass plate

For improving the accuracy of the microarray and for short RNA target analysis, we analysed the hybridisation and base discrimination abilities of the microarray mounted with the 2′-*O*-methyl-RNA probe incorporating s^2Um. 5′-Biotiniylated 2′-*O*-methyl-RNAs or DNAs were immobilised on a streptavidin coated slide glass plate. The target oligonucleotide sequences of the microarray used a short miRNA 22mer sequence which is known as let7 miRNA.[29] These target oligonucleotides were labelled with Cy3 at the 5′-end. Hybridisation of probes and target oligonucleotides was performed for 2 h at ambient temperature in a 10 mM sodium phosphate buffer at pH 7.0 containing 150 mM NaCl. After the hybridisation and the subsequent washing of the microarray, the fluorescent intensity of each hybridised spot was determined by the average of 15 spots randomly sampled from 64 hybridised spots. The fluorescent intensities of the s^2Um-containing microarray and typical hybridised spot images are shown in Figure 8. In the case of the 2′-*O*-methylated RNA probe containing Um, the fluorescent intensity of the Um–A Watson–Crick type base pair and the Um–G wobble type base pair was almost at the same level. On the other hand, in the case of a 2′-*O*-methylated RNA probe incorporating s^2Um, the difference in fluorescent intensity between the s^2Um–A Watson–Crick type base pair and the s^2Um–G wobble type base pair was clearly observed. Moreover, when the fluorescent intensity of the 2′-*O*-methylated RNA probe containing Um was compared with that of the 2′-*O*-methylated RNA probe containing s^2Um, the intensity of the latter having a s^2Um–A mismatched base pair was much higher than that of the former with a Um–A mismatched base pair. Thus, the 2′-*O*-methylated RNA probe with s^2Um proved to be superior to that with Um.

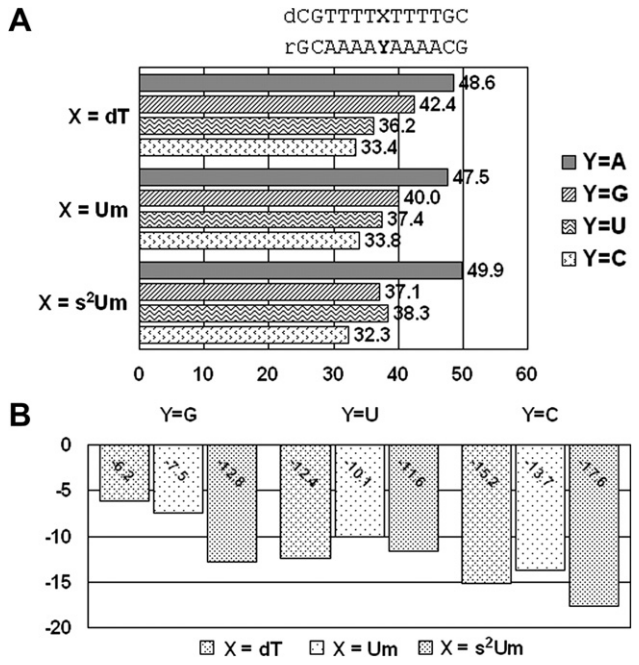

Figure 7. T_m analysis of DNA/RNA duplexes containing 2′-*O*-methyl-2-thiouridine (s^2Um). (A) Melting temperatures of duplexes formed between oligodeoxyribonucleotides containing s^2Um, Um or dT and their complementary RNA strand having fully matched or single mismatched sight at counterpart of modified base. Duplex sequences are shown above the chart. Melting temperature experiments were performed under following conditions: Sodium phosphate buffer at pH 7.0 containing 1 M NaCl). (B) Base discrimination of dT, Um or s^2Um in the DNA/RNA duplexes. Values determined from T_m differences of fully matched and single mismatched DNA/RNA duplexes.

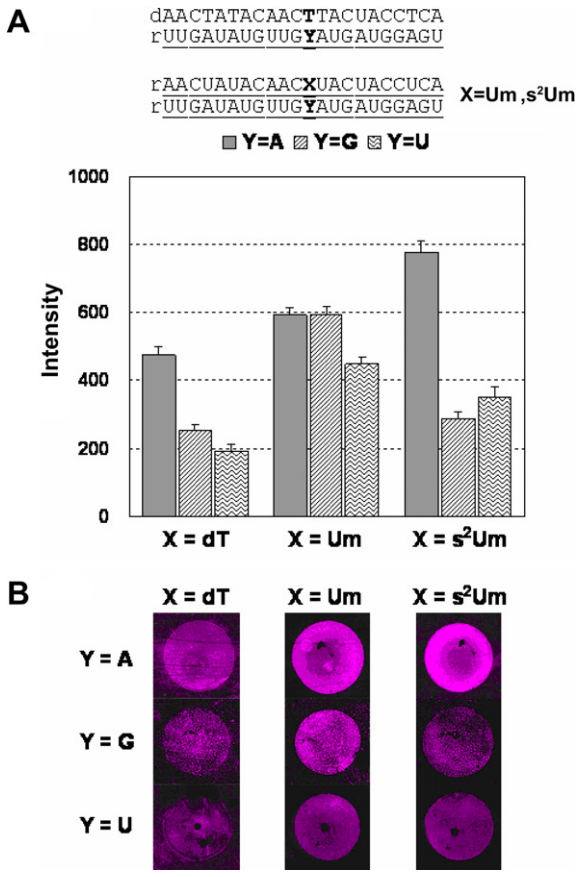

Figure 8. Hybridisation and single base mismatch detection abilities of s^2Um-containing probes on slide glass plates. (A) Sequences and fluorescent intensities of the 2′-*O*-methylated RNA and DNA microarray. Fluorescent intensity of each oligonucleotide probe determined by average of 15 spots randomly sampled from 64 hybridised spots. 2′-*O*-Methyl RNA is underlined. (B) Fluorescent images of typical spots of fully matched and wobble type mismatched spots.

3. Discussion

We examined the hybridisation and base discrimination abilities of oligonucleotides containing s^2Um. Our experiments revealed that oligonucleotides containing s^2Um enhanced the stabilities of duplexes and exhibited selective recognition ability towards the adenine base when hybridised with the complementary RNA strands. In particular, in the case of a wobble base pair, the extent of destabilisation of the s^2Um–G base pair was greater than that of the Um–G and dT–G base pairs. These results were consistent with the hybridisation properties of oligonucleotides having a 2-thiouracil base previously reported.[24,25] Moreover, in the case of DNA/DNA duplexes containing s^2Um, the extent of destabilisation of the s^2Um–dG base pair was greater than that of the s^2Um–dC or the s^2Um–dT base pairs (Fig. 1B). However, in the base discrimination ability of DNA/DNA duplexes in 1 M NaCl conditions, in spite of incorporating modified bases, was to the same extent as that of dT, Um or s^2Um (Fig. 6B). It is not clear why the differences between dT, Um and s^2Um disappeared under a 1 M NaCl condition. Perhaps, the high concentration of sodium cations induced the extremely stabilised B-form DNA structure and the differences that arose from structural distortion around the mismatched base pair region were hardly discernible from the effects of the modified base because the differences in base discrimination between the modified and unmodified DNA/DNA duplexes disappeared. On the other hand, when oligonucleotides containing s^2Um were hybridised with an RNA strand, the sodium cation concentration did not affect their base discrimination ability (Figs. 2, 4, 7B). The superior base discrimination ability of oligonucleotides containing s^2Um towards the complementary RNA strands was probably due to the diversity of the RNA structure. It is well known that RNA forms various types of non-canonical base pairs.[30–33] These non-canonical base pairs were sometimes stabilised by hydrogen bonding with the oxygen atom at the 2′ position via some water molecules.[30,33] Possibly, the water molecules which were involved in water-mediated hydrogen bonding in the wobble base pair region were fixed strongly and were not dehydrated by sodium cations. This observation was based on the fact that the T_m difference of the wobble base pair was maintained. In addition, the oligonucleotide sequence used in our experiments 2′-*O*-methylated RNA/DNA duplexes containing an Um–dT or s^2Um–dT mismatch base pair exhibited unusual stabilisation of the duplexes (Fig. 3). In particular, the Um–dT mismatch base pair was more stable than the Um–dA Watson–Crick base pair. It was reported that an RNA/RNA duplex

containing a U–U mismatch base pair was stabilized when the mismatch base pair was located near the helix end.[34] On the other hand, it was reported that an RNA/DNA duplex containing a U–dT mismatch base pair at the centre position of the helix was not so dramatically stabilised.[35] According to these studies of the hybridisation property of duplexes containing U–U or U–dT mismatch base pairs, it was expected that the sequences containing the Um–dT or s^2Um–dT mismatch base pairs used in this study might not stabilise the duplexes so much, because the positions of these mismatch base pairs were located at the centre of the helixes. The reason why the Um–T and s^2Um–T mismatched base pairs were significantly stable was unclear. Therefore, further studies are needed.

The fluorescent intensity analysis of a microarray having the s^2Um-containing probes revealed that these probes could improve the intensity and accuracy of microarrays. In particular, Um-containing probes could not discriminate A from G. On the contrary, s^2Um exhibited almost the same normal complementary base. These results agree well with the T_m analysis of s^2Um-containing duplexes. Hence, the incorporation of s^2Um into microarray probe oligonucleotides was useful for improving the signal intensity and preventing undesirable mismatch hybridisation. Similarly, utilisation of s^2Um for a microarray was demonstrated by Kierzek et al.[36] They reported the use of s^2Um-containing short probes for predicting the secondary structure of rRNA. Their results revealed that s^2Um-containing probes could improve prediction of the secondary structure because of the accurate and stable hybridisation abilities of s^2Um. In conclusion, oligonucleotides containing s^2Um exhibited excellent base discrimination ability. The stability of the duplexes and their base discrimination ability towards the complementary RNA strands were superior to those of the complementary DNA strands. Microarray experiments revealed that s^2Um is a potent modified nucleoside, which can be used to improve the accuracy and intensity of the microarray.

4. Experimental

4.1. Materials

All DNA oligonucleotides containing natural nucleotides were purchased from Sigma Genosys Japan, Inc., as lyophilized powders, which were purified by cartridge purification. RNA oligonucleotides containing natural nucleotides were purchased from Fasmac, Inc., as lyophilised powders, which were purified by RP-HPLC. Commercially available phosphoramidites were purchased from Glen Research, Inc.

4.2. Synthesis and purification of modified oligonucleotides

Modified oligoribonucleotides were synthesised on an Applied Biosystems 382 DNA/RNA synthesiser, using β-cyanoethyl phosphoramidite chemistry, and purified using the DMTr-on mode. For the synthesis of 2′-*O*-methyl RNA oligonucleotides, 2′-*O*-methylnucleotide-3′-*O*-phosphoramidite derivatives were used (Glen Research). The 3′-*O*-phosphoramidites of 2′-*O*-methyl-2-thiouridine (s^2Um) were synthesised according to published procedures.[26] A 0.1 M solution of each phosphoramidite in anhydrous acetonitrile was used for the synthesis of oligonucleotides containing s^2Um. For the incorporation of s^2Um into oligonucleotides, a coupling time of 10 min was used. The default setting was used for all other steps of the oligonucleotide synthesis protocol. Oxidation of internucleotidic phosphite intermediates to phosphate derivatives was performed using a commercially available 0.02 M iodine solution of pyridine/water/THF (Glen Research) with a reaction time of 15 s for oxidation.[37] After completing the synthesis, a solid support was suspended in aqueous ammonium hydroxide/EtOH (3:1, v/v) and kept at room temperature for 2 h. The solid support was then filtered, and the filtrate was kept at room temperature for 10 h to complete the removal of all protecting groups. The crude oligonucleotides were purified over Sep-pak C18 cartridges (waters). After cartridge purification, the purity of the desired oligonucleotides was confirmed by RP-HPLC.

4.3. Melting temperature (T_m) measurement

UV-melting curves were measured on a Shimadzu UV-1700 UV/vis spectrophotometer equipped with a temperature controller and a multi cell Peltier block. A temperature gradient of 0.5 °C/min was applied and a heating–cooling–heating cycle was used. The cell compartment was flushed with dry air to prevent the condensation of water on the cuvettes. In all the cases, heating and cooling curves were superimposable which indicated reversible equilibrium conditions. T_m data were defined as the maxima of the first order derivative of the melting curves and were shown to correspond to ±1 °C of those determined at half of the maximal hyperchromicity after baseline correction.

4.4. Fabrication of 2′-*O*-methyl-RNA microarray

For the synthesis of probe oligonucleotides used in microarray experiments, a phosphoramidite derivative was used for introducing a biotin moiety into the 5′ terminal hydroxyl group (Glen Research). The 5′-biotinylated oligonucleotides were synthesised and purified using a procedure similar to that described for the synthesis of modified oligonucleotides. A solution of each 5′-biotynylated probe oligonucleotides (50 μM) in DMSO/H_2O (1:1, v/v) was spotted on a streptavidin coated slide glass plate (Greiner) using a Greiner Manual Microarrayer and Manual Spotter (Greiner). After the spotting of oligonucleotides using the probes, the spots on the slide glass plate were air-dried at ambient temperature for 24 h and then the slide glass plate was dipped into distilled water for 3 min to remove excess probe oligonucleotides. After dipping, the water remaining on the slide glass plate was blown off by an air duster. The slide glass plate was stored in a refrigerator until just before the microarray experiments.

4.5. Microarray experiments

For the synthesis of target oligonucleotides used in the microarray experiments, a phosphoramidite reagent was used for introducing a Cy3 fluorescent group into the 5′-terminal hydroxyl group (Glen Research). The 5′-Cy3 labelled oligonucleotides were synthesised and purified using a procedure similar to that used in the synthesis of modified oligonucleotides. Each 5′-Cy3 labelled target oligonucleotide (100 μM) was dissolved in a 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl. Hybridisation was performed at 24 °C for 2 h in a Hybridisation Chamber TX710 (TaKaRa) with a Spaced Cover Glass L (TaKaRa). After hybridisation, the slide glass plate was dipped into a 1× SSC buffer for 1 min to remove the cover glass and the excess target oligonucleotides. Furthermore, the slide glass plate was rinsed twice with a 0.2× SSC buffer for 1 min. Hybridisation spots were observed using a fluorescent microscope (OLYMPUS). The same exposure time setting was used for capturing all fluorescent images.

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