Sequence-Specific Hybridization between Two Different Types of Peptide Nucleic Acids

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Nielsen's-type peptide nucleic acids (PNA) and the oxy-PNA (OPNA), both carrying thymine or adenine bases, were prepared. Hybridizations were observed not only between the complementary pairs of PNA(A_{12})–DNA(T_{12}) and OPNA(A_{12})–DNA(T_{12}), but also between the complementary pairs of the artificial nucleic acid analogues, OPNA(A_{12})–PNA(T_{12}). In the three-component mixture of PNA(T_{12})–DNA(T_{12})–OPNA(A_{12}), OPNA(A_{12}) was found to hybridize preferentially with DNA(T_{12}). Addition of DNA(T_{12}) to a preformed duplex of OPNA(A_{12})–PNA(T_{12}) gave rise to a strand displacement to form a OPNA(A_{12})–DNA(T_{12}) duplex. No triplex formation was detected in the three-component mixture.

Peptides carrying nucleobases on the side chain have been attracting interest as antisense molecules that may be used for diagnostic and medicinal uses.[1] Since DNAs and RNAs have nucleobases at every six main-chain atoms, the peptides must consist of δ-amino acids carrying a single nucleobase or must be alternating peptides carrying single nucleobase in every dipeptide unit. The alternating peptides have been synthesized by several workers,[2–5] but their hybridization with DNAs has not been reported, probably due to insufficient flexibility of the peptide main chain. Nielsen and co-workers showed that peptides of δ-amino acids H_2N–CH_2–CH_2–N(–CO–CH_2–B)–CH_2–COOH (B = nucleobases), hybridize with complementary DNAs more strongly than the complementary DNA–DNA pairs.[6] The peptides have been named as peptide nucleic acids (PNAs), although they do not carry acid groups. After the success of Nielsen's PNA, several groups reported PNAs of different types of δ-amino acids.[7] However, most of these PNAs failed to hybridize with DNAs, probably due to mismatching of the structure.

Very recently, we have shown that peptides of another type of δ-amino acid H_2N–C*H(–CH_2–CH_2–B)–CH_2–O–CH_2–COOH, hybridize with complementary DNAs.[8,9] The melting curve of the hybridized complex showed all-or-none-type transition that enables detection of even a single mismatch in the base sequences. Since the latter δ-amino acid has an ether linkage in the main chain, the peptide has been named as oxy-peptide nucleic acid (OPNA). The success of the OPNA may be due to the presence of the ether linkages that provide enough flexibility in the peptide main chain.

In this article, the two types of peptide nucleic acids, i.e., Nielsen's PNA (I) (simply called as PNA hereafter) and the OPNA (II) carrying adenine (A) or thymine (T) nucleobases, were compared in their hybridization behavior with the complementary DNAs (III) (Chart 1). Furthermore, hybridization between the complementary pair of PNA and OPNA was also investigated to examine the consistency of the nucleic acid analogues.

Experimental

Preparation of the OPNA-type δ-amino acids with A and T nucleobases has been described before.[9] The dodecapeptides of the PNA-type amino acids and the OPNA-type amino acids were synthesized through solid-phase peptide synthesis.[8] An Fmoc–NH–SAL–PEG resin (super acid-labile polyethylene glycol resin from Watanabe Chemicals, Hiroshima, Japan) was used as a support and benzotriazole-1-yloxytripyrrolidinylphosphonium hexaflurophosphate (PyBop)/HOBt as the coupling reagents. First, an $^{\varepsilon}N$-Boc–lysine was linked to the resin to improve solubility and the coupling of the Fmoc–δ-amino acid was repeated 12 times. The benzoyl protecting groups of adenine units were removed by treat-

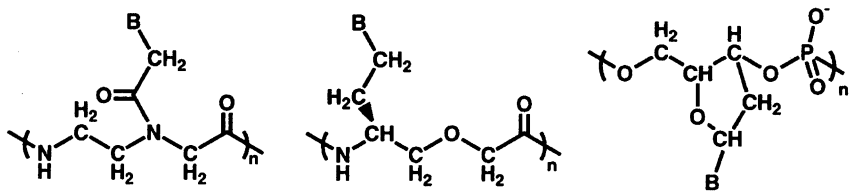

Chart 1.

ing the resin with ethylenediamine. The peptides were cleaved off from the resin by TFA containing 20% *m*-cresol. The crude PNA and OPNA were purified by a reverse-phase HPLC (C18 column) with a linear gradient of 7—32% of eluent A (0.1% TFA in acetonitrile) in eluent B (0.1% TFA in water) over 50 min at a flow rate of 5 mL min^{-1}. DNA(A_{12}) and DNA(T_{12}) were purchased from Life Technologies Oriental, Inc. (Tokyo, Japan).

Melting curves were recorded on a JASCO Ubest55 instrument equipped with a computer-controlled thermostated cell holder (JASCO ETC505). Absorbance at 260 nm of a mixture containing 1.8 μmol dm^{-3} of duplex or equivalent was measured at an interval of 0.5 °C with heating or cooling rate of 0.5 °C min^{-1} or 1 °C min^{-1}. CD spectra were recorded on a JASCO J-720 WI instrument.

Results and Discussion

Hybridizations of the DNA(T_{12})–DNA(A_{12}), DNA(T_{12})–PNA(A_{12}), and DNA(T_{12})–OPNA(A_{12}) Pairs. Sequence-specific hybridizations have been reported for the above three pairs previously.[8] In all cases, UV absorbance at 260 nm plotted against mole percent of the adenine component (Job plot) showed a minimum at the 1 : 1 ratio (Fig. 1). The melting points T_m, were 30 °C for DNA(T_{12})–DNA(A_{12}) pair, 55 °C for DNA(T_{12})–PNA(A_{12}) pair, and 43 °C for DNA(T_{12})–OPNA(A_{12}) pair.[8] These melting temperatures have been measured at a duplex concentration of 5.9 μmol dm^{-3} and can be transformed to the T_m values at 1.8 μmol dm^{-3} by using Eq. 1:[10]

$$1/T_m = (2.3R/\Delta H^\circ)\log c_T + \Delta S^\circ/\Delta H^\circ, \quad (1)$$

where ΔH° and ΔS° are the standard enthalpy and entropy of dissociation of the duplex[8] and c_T is the total strand concentration.

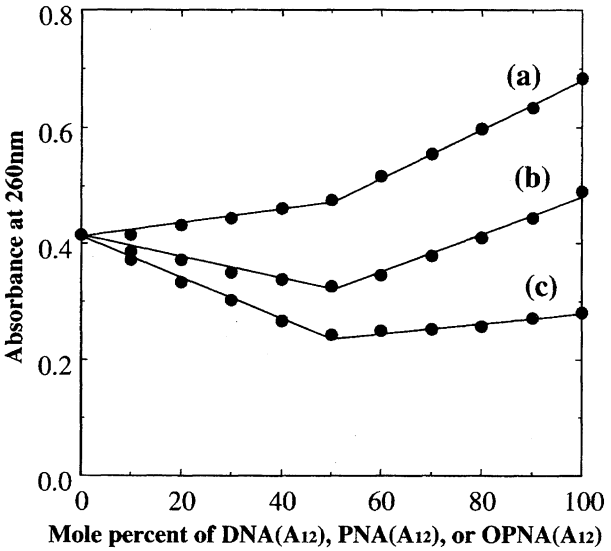

Fig. 1. Job plots for the absorbance at 260 nm of the mixtures of (a) DNA(T_{12})–DNA(A_{12}), (b) DNA(T_{12})–PNA(A_{12}), and (c) DNA(T_{12})–OPNA(A_{12}), in 150 mmol dm^{-3} NaCl, 10 mmol dm^{-3} NaH_2PO_4 and 0.1 mmol dm^{-3} EDTA at pH 7.0 and 25 °C. Total base concentration = 45 μmol dm^{-3}. Optical path length = 1 cm.

The corrected melting temperatures are: 27 °C for DNA(T_{12})–DNA(A_{12}), 52 °C for DNA(T_{12})–PNA(A_{12}), and 41 °C for DNA(T_{12})–OPNA(A_{12}). The melting points are listed in Table 1.

The heating (dissociation) curves for the three 1 : 1 mixtures were virtually identical to the cooling (re-association) curves. The reversible melting curves indicate that rapid equilibrium is attained between the single-stranded and double-stranded form for the three mixtures through rapid conformational change of DNA, PNA, and OPNA.

Hybridization between PNA(T_{12}) and DNA(A_{12}). Hybridization between PNA(T_{12}) and DNA(A_{12}) has been reported.[6b] The stoichiometry of the PNA(T_{12})–DNA(A_{12}) complex was 2 : 1, indicating that two PNA(T_{12}) and one DNA(A_{12}) form a triplex. The melting curve of the 1 : 1 mixture of PNA(T_{12}) and DNA(A_{12}) is shown in Fig. 2.[11] The heating curve shows no clear plateau below 90 °C, but the cooling curve shows a melting point T_m at 58 °C. The hysteresis did not depend on the heating/cooling rates over the range 0.5—1 °C min^{-1}. Similar hysteresis has been reported for a 2 : 1 mixture of pyrimidine-rich PNA and the complementary DNA and explained in terms of a slow conformational change of PNA.[12]

Hybridization between PNA(T_{12}) and PNA(A_{12}). Hybridization between PNA(T_{12}) and PNA(A_{12}) was also studied. The Job plot indicates a 2 : 1 complex of PNA(T_{12}) and PNA(A_{12}) [Fig. 3, data (a)]. In the figure, no trough at the 1 : 1 ratio is detected, indicating that the 2 : 1 complex is the only possible structure in the mixture. The preference of triplex formation of pyrimidine-rich PNA is common to DNA and PNA. The melting curve of the PNA(T_{12})–PNA(A_{12}) 1 : 1 mixture showed a small hysteresis with a T_m >83 °C on heating and ca. 70 °C on cooling (Fig. 4).[11]

Hybridization between PNA(T_{12}) and OPNA(A_{12}). Hybridization between PNA(T_{12}) and OPNA(A_{12}) was also examined. The Job plot for the PNA(T_{12})–OPNA(A_{12}) mixtures is shown in Fig. 3 [data (b)]. The minimum at the 1 : 1 molar ratio indicates duplex formation between the two types of nucleic acid analogs, despite the fact that the PNA-

Table 1. Summary of the Hybridization Properties of the DNA, PNA, and OPNA Carrying Adenine and Thymine Bases

Melting temperatures (°C) and stoichiometric ratios. Duplex Concentration = 1.8 μmol dm^{-3}.

	DNA(A_{12})	PNA(A_{12})	OPNA(A_{12})
DNA(T_{12})[a)]	27 (heating and cooling) (1 : 1 duplex)	52 (heating and cooling) (1 : 1 duplex)	41 (heating and cooling) (1 : 1 duplex)
PNA(T_{12})	> 80 (heating) ≈ 58 (cooling) (2 : 1 triplex)	> 83 (heating) ≈ 70 (cooling) (2 : 1 triplex)	> 80 (heating) ≈ 35 (cooling) (2 : 1 triplex)

a) Melting temperatures were measured at the duplex concentration of 5.9 μmol dm^{-3} and transformed to the values at 1.8 μmol dm^{-3} by using Eq. 1 in the text.

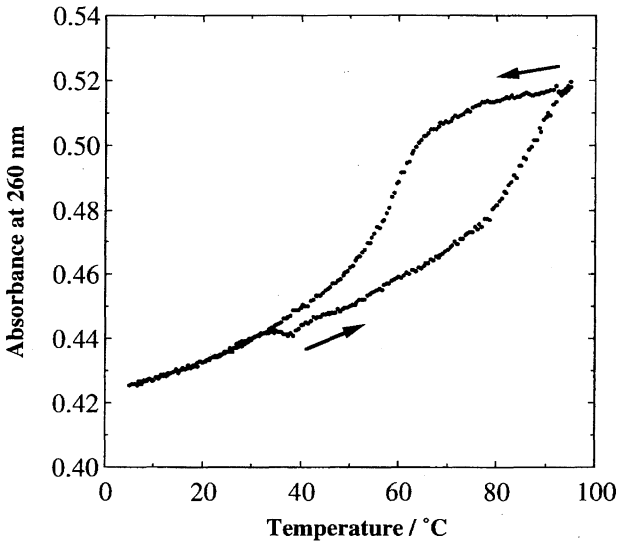

Fig. 2. Temperature dependence of absorbance at 260 nm for equimolar mixtures of PNA(T_{12})–DNA(A_{12}) in 150 mmol dm^{-3} NaCl, 10 mmol dm^{-3} NaH_2PO_4 and 0.1 mmol dm^{-3} EDTA, pH 7.0. Duplex concentration = 1.8 μmol dm^{-3}. The melting curves were recorded at an interval of 0.5 °C/0.5 min.

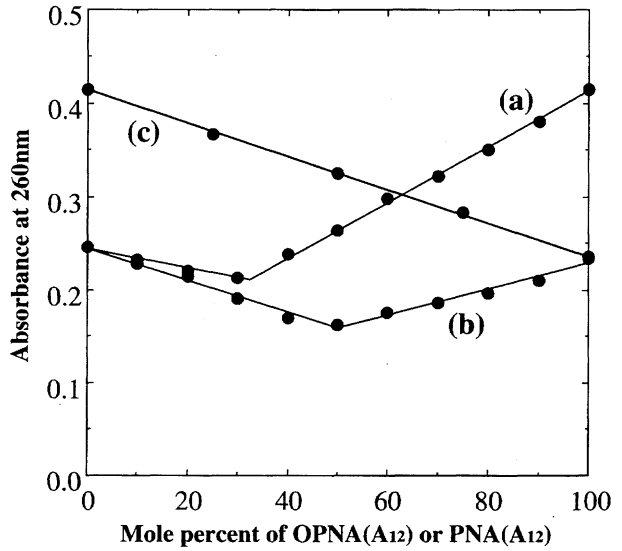

Fig. 3. Job plots for the absorbance at 260 nm of the mixtures of (a) PNA(T_{12})–PNA(A_{12}), (b) PNA(T_{12})–OPNA(A_{12}), and (c) PNA(A_{12})–OPNA(A_{12}) in 150 mmol dm^{-3} NaCl, 10 mmol dm^{-3} NaH_2PO_4 and 0.1 mmol dm^{-3} EDTA at pH 7.0 and 5 °C. Total base concentration = 38 μmol dm^{-3}. Optical path length = 1 cm.

(T_{12})–DNA(A_{12}) and PNA(T_{12})–PNA(A_{12}) mixtures form a triplex with a 2 : 1 ratio. The preference of the duplex rather than the triplex is compatible with the finding that the OPNA(A_{12}) forms only a duplex with DNA(T_{12}). These results indicate that OPNAs favor duplex rather than triplex, whereas pyrimidine-rich PNAs favor triplex.

Absorption intensities of PNA(A_{12})–OPNA(A_{12}) mixtures are also shown in Fig. 3 [data (c)]. The absence of hypochromicity in the non-complementary mixture indicates

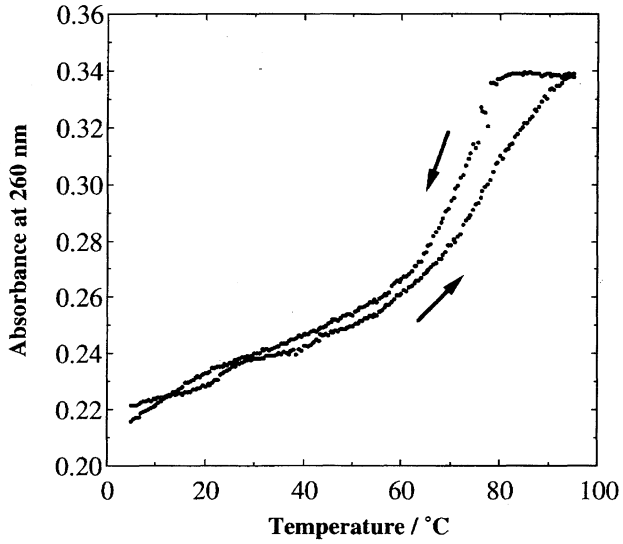

Fig. 4. Temperature dependence of absorbance at 260 nm for equimolar mixtures of PNA(T_{12})–PNA(A_{12}) in 150 mmol dm^{-3} NaCl, 10 mmol dm^{-3} NaH_2PO_4 and 0.1 mmol dm^{-3} EDTA, pH 7.0. Duplex concentration = 1.8 μmol dm^{-3}. The melting curves were recorded at an interval of 0.5 °C/0.5 min.

that the hybridization is sequence-specific.

Hybridization between the two nucleic acid analogs, PNA(T_{12}) and OPNA(A_{12}), is the first example for artificial nucleic acid analogues to hybridize with sequence specificity. The hybridization indicates either that the duplexes from DNA–DNA, DNA–PNA, DNA–OPNA, PNA–PNA, and PNA–OPNA pairs take similar conformations or that the OPNA is so flexible as to adapt to different duplex structures. Figure 5 compares CD spectra of the 4 duplexes with A–T pairs. Since PNA is achiral, no CD is expected for the PNA(T_{12})–PNA(A_{12}) duplex. Strong CD spectra were observed for the duplexes of DNA(T_{12})–DNA(A_{12}), DNA(T_{12})–PNA(A_{12}), and DNA(T_{12})–OPNA(A_{12}), but their profiles are significantly different from each other. The strong CD suggests double-helical structures for the three duplexes, but the duplex structures must be significantly different from each other.

No CD was observed for the OPNA(A_{12})–PNA(T_{12}) duplex, indicating no regular helical structure for the duplex, despite the fact that the OPNA(A_{12})–DNA(T_{12}) duplex must have a regular double-helical structure. The above results indicate that OPNA(A_{12}) has a very flexible chain and can adapt to form duplex with DNA and take a double-helical structure. The duplex with achiral PNA has a non-helical structure.

A marked hysteresis was observed in the melting curve of the PNA(T_{12})–OPNA(A_{12}) mixture (Fig. 6). A higher T_m (> 80 °C) was observed on heating and a lower T_m (ca. 35 °C) on cooling. The marked hysteresis indicates that the duplex once formed is difficult to dissociate and the dissociated pair has difficulty to form the duplex.

DNA(T_{12})–PNA(T_{12})–OPNA(A_{12}) Three-Component Mixtures with Different Orders of Mixing. To com-

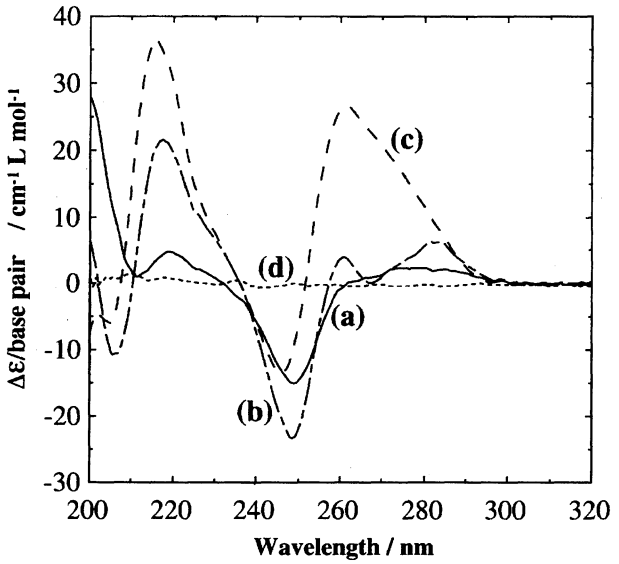

Fig. 5. CD spectra of the duplexes with A–T complementary base pairs. (a: —) DNA(T_{12})–OPNA(A_{12}), (b: – · –) DNA-(T_{12})–DNA(A_{12}), (c: - - -) DNA(T_{12})–PNA(A_{12}), and (d: ····) PNA(T_{12})–OPNA(A_{12}) in 150 mmol dm^{-3} NaCl, 10 mmol dm^{-3} NaH_2PO_4 and 0.1 mmol dm^{-3} EDTA at pH 7.0 and 5 °C. Total base pair concentration = 68 μmol dm^{-3}. Optical path length = 2 mm.

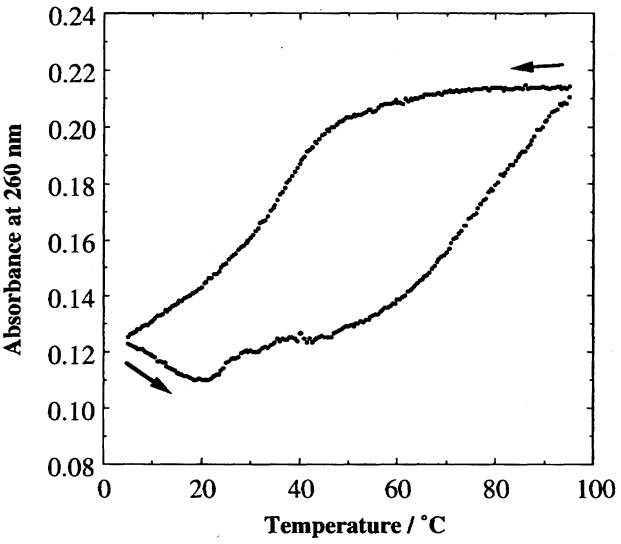

Fig. 6. Temperature dependence of absorbance at 260 nm for equimolar mixtures of PNA(T_{12})–OPNA(A_{12}) in 150 mmol dm^{-3} NaCl, 10 mmol dm^{-3} NaH_2PO_4 and 0.1 mmol dm^{-3} EDTA, pH 7.0. Duplex concentration = 1.8 μmol dm^{-3}. The mixture was stored for 1 h after mixing and subjected to a thermocycle of 95 °C to 5 °C. The melting curve was recorded at an interval of 0.5 °C/0.5 min.

pare relative stabilities of the duplexes and to see a possible triplex formation, melting curves of the DNA(T_{12})–PNA-(T_{12})–OPNA(A_{12}) 1 : 1 : 1 mixtures were measured with different orders of mixing. First, OPNA(A_{12}) was added to the mixture of DNA(T_{12})–PNA(T_{12}). Since no interaction is expected in the DNA(T_{12})–PNA(T_{12}) mixture, this mixing process corresponds to a concomitant mixing of the three components. The CD spectrum of the three-component mixture was coincident with that of the DNA(T_{12})–OPNA(A_{12}) duplex. The melting curve also agreed with that of the latter duplex [Fig. 7, data (a)], indicating that only DNA-(T_{12})–OPNA(A_{12}) duplex is formed in the three-component mixture and that the DNA(T_{12})–OPNA(A_{12}) duplex is more rapidly formed than the PNA(T_{12})–OPNA(A_{12}) duplex. This result is consistent with the melting curves of DNA-(T_{12})–OPNA(A_{12})[8] and of PNA(T_{12})–OPNA(A_{12}) (Fig. 6) and the melting temperatures collected in Table 1. Since the T_m on heating is 41 °C for DNA(T_{12})–OPNA(A_{12}) duplex and >80 °C for PNA(T_{12})–OPNA(A_{12}), the preformed DNA(T_{12})–OPNA(A_{12}) duplex will melt at 41 °C. The dissociated component will form again a DNA(T_{12})–OPNA(A_{12}) duplex, because its T_m on cooling (41 °C) is higher than that of the PNA–OPNA duplex (ca. 35 °C). The above result clearly shows that the DNA(T_{12})–OPNA(A_{12}) duplex is easier to form and easier to dissociate than the PNA-(T_{12})–OPNA(A_{12}) duplex.

In the second experiment, DNA(T_{12})–OPNA(A_{12}) duplex was first formed and after keeping the solution for 1h at 25 °C, an equivalent amount of PNA(T_{12}) was added. The CD spectrum after storing the three-component mixture at 25 °C for 1 h, was virtually identical to that of DNA(T_{12})–OPNA-(A_{12}) duplex, indicating that neither the strand displacement

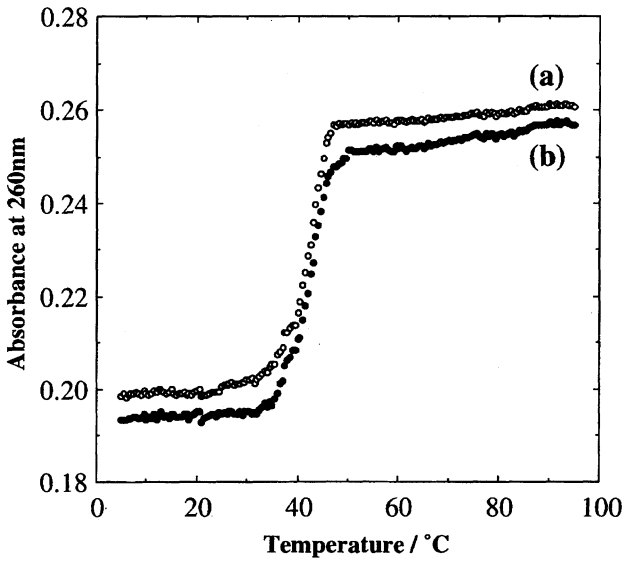

Fig. 7. Temperature dependence of absorbance at 260 nm for three-component mixtures of DNA(T_{12})–PNA-(T_{12})–OPNA(A_{12}) (1 : 1 : 1) in 150 mmol dm^{-3} NaCl, 10 mmol dm^{-3} NaH_2PO_4 and 0.1 mmol dm^{-3} EDTA, pH 7.0. Total base concentration = 43 μmol dm^{-3}. (= 1.2 μmol dm^{-3}duplex + equivalent mole of single strand). The melting curves were recorded at an interval of 0.5 °C/0.5 min. (a) OPNA(A_{12}) was added to the mixture of DNA-(T_{12})–PNA(T_{12}). The melting curve was measured 3 h after the mixing of the OPNA(A_{12}). (b) PNA(T_{12}) was added to a performed duplex of DNA(T_{12})–OPNA(A_{12}) after storing the duplex at 25 °C for 2 h. The melting curve was measured after storing the three-component mixture for further 1 h.

of the duplex from DNA–OPNA to DNA–PNA nor the triplex formation took place. The melting curve [Fig. 7, data (b)] was identical to that of the DNA(T_{12})–OPNA(A_{12}) duplex and no hysteresis was observed.

Strand Displacement from PNA(T_{12})–OPNA(A_{12}) Duplex to DNA(T_{12})–OPNA(A_{12}) Duplex. In the third experiment, a PNA(T_{12})–OPNA(A_{12}) duplex was preformed and the mixture was stored for 30 min at 25 °C. Then an equivalent amount of DNA(T_{12}) was added. After addition of DNA(T_{12}), the melting curve was measured, as shown in Fig. 8 [data (a)]. During the first heating process, the melting curve showed a gentle slope with the melting temperature around 60 °C, suggesting that the dissociation of PNA(T_{12})–OPNA(A_{12}) duplex is the major event. After dissociation at elevated temperature, the three-component mixture behaved similarly to the above three-component mixtures and showed the melting profile of DNA(T_{12})–OPNA(A_{12}) duplex. The CD spectrum after the thermocycle also indicated the strand displacement from PNA(T_{12})–OPNA(A_{12}) duplex to DNA(T_{12})–OPNA(A_{12}) duplex.

When DNA(T_{12}) was added to a preformed PNA(T_{12})–OPNA(A_{12}) duplex after storing the latter solution at 25 °C for 2 h, no strand displacement was observed, as seen from the melting curves in Fig. 8 [data (b)]. In the latter curves, temperature dependence was observed neither on heating up to 95 °C nor cooling down to 5 °C. The absence of any temperature dependence indicates that the PNA(T_{12})–OPNA(A_{12}) duplex changed its conformation to a more compact form during the storage for 2 h and the latter cannot allow strand displacement. It is plausible that the PNA(T_{12})–OPNA(A_{12}) duplex initially takes a "loose" conformation that allows a strand displacement with DNA(T_{12}). The loose conformation turns into a more compact conformation during the storage for 1—2 h and the compact form does not allow the strand displacement. The conformational change from the "loose" duplex of PNA(T_{12})–OPNA(A_{12}) to the compact form is also suggested from a small decrease of absorbance at 260 nm.

No triplex was detected in the DNA(T_{12})–OPNA(A_{12})–PNA(T_{12}) equimolar mixtures, despite the fact that triplex formations have been found in the PNA(T_{12})–DNA(A_{12}) and PNA(T_{12})–PNA(A_{12}) 2 : 1 mixtures.

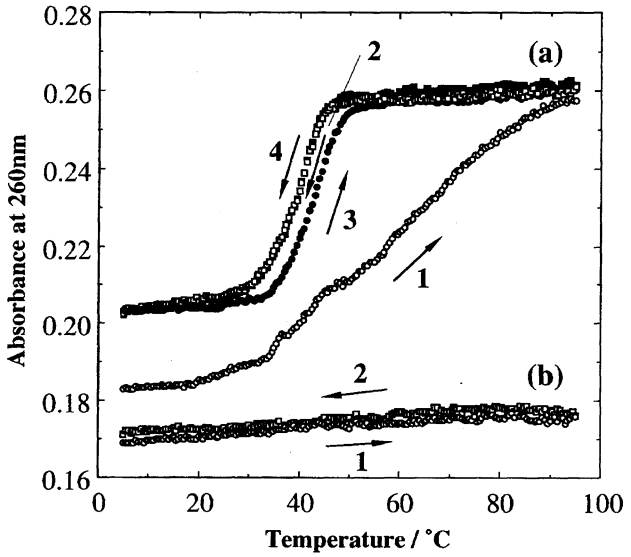

Fig. 8. Temperature dependence of absorbance at 260 nm for three-component mixtures of DNA(T_{12})–PNA(T_{12})–OPNA(A_{12}) (1 : 1 : 1) in 150 mmol dm^{-3} NaCl, 10 mmol dm^{-3} NaH_2PO_4 and 0.1 mmol dm^{-3} EDTA, pH 7.0. Total base concentration = 43 μmol dm^{-3} (= 1.2 μmol dm^{-3} duplex + equivalent mole of single strand). The melting curves were recorded at an interval of 0.5 °C/0.5 min. (a) DNA(T_{12}) was added to a performed duplex of PNA(T_{12})–OPNA(A_{12}) that was stored for 30 min at 25 °C. The melting curve was measured immediately after the mixing of the third component. (b) DNA(T_{12}) was added to the performed duplex after storing the latter at 25 °C for 2 h. The melting curve was measured after storing the three-component mixture for further 1 h.

Conclusions

Hybridization properties of DNAs, PNAs, and OPNAs carrying A or T nucleobases were compared in Table 1. Hybridization was observed not only in the DNA(T_{12})–PNA(A_{12}) and DNA(T_{12})–OPNA(A_{12}) pairs but also in the PNA(T_{12})–OPNA(A_{12}) pair. The sequence-specific hybridization between the two artificial nucleic acid analogues was first demonstrated in this study. The flexibility of the OPNA chain may be responsible to the duplex formations with both DNA and PNA. The OPNA(A_{12}) forms duplex more preferentially with DNA(T_{12}) than with PNA(T_{12}). This was most clearly demonstrated by a strand displacement from PNA(T_{12})–OPNA(A_{12}) duplex to DNA(T_{12})–OPNA(A_{12}) duplex.

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11 Although the stoichiometry of the complex is 2 : 1, the melting curves of PNA(T_{12})–DNA(A_{12}) and PNA(T_{12})–PNA(A_{12}) mixture were measured at the 1 : 1 ratio for comparison with the melting curves of the 1 : 1 : 1 three-component mixtures.

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