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Affinity gel electrophoresis of nucleic acids

Nucleobase-selective separation of DNA and RNA on agarose-poly(9-vinyladenine) conjugated gel

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ABSTRACT

Poly(9-vinyladenine) (PVAd) was immobilized within an agarose gel matrix to produce a novel affinity gel for the base-specific separation of nucleic acids by electrophoresis. The shape (single- or double-stranded) and base content of nucleic acids were specifically recognized by the affinity gel. Only single stranded DNA, of which the sequence is not regular enough to form a stable duplex hairpin structure, was selectively adsorbed over double-stranded DNA. Among five polynucleotides having different bases such as poly(A), poly(G), poly(C), poly(U) and poly(I), poly(U) and poly(I) were base-specifically adsorbed by PVAd, probably by hydrogen bond formation. The effect of the molecular mass and size of poly(9-vinyladenine) was also examined.

INTRODUCTION

The separation and purification of nucleic acids on the basis of size, shape, base composition and base sequence are very important in gene technology and related fields. The most commonly used techniques for this purpose are standard agarose or polyacrylamide gel electrophoresis [1] and high-performance liquid chromatography (HPLC) [2,3], which mainly offer separation based on differences in size or chain length; a smaller molecule migrates through the matrices faster than a larger molecule. On the other hand, affinity gel electrophoresis (AGE) [4] and high-performance affinity chromatography (HPAC) [5] are attractive techniques for the specific base and sequence recognition of nucleic acids, and affinity ligands such as poly(uridylic acid) [poly(U)], one strand of polynucleotides, and oligonucleotides are immobilized on supports such as cellulose, agarose and silica gel for the specific separation of the complementary strand in mixtures of polynucleotides [6–8]. These affinity materials will resolve nucleic acids with high specificity, but there is a defect in the stability of nucleic acids immobilized on a support. Nucleic acids are decomposed by enzyme-catalysed hydrolysis and are not suitable as affinity ligands for electrophoresis because of their anionic character. Therefore, synthetic neutral analogues having nucleic acid bases such as vinyl polymer analogues of nucleic acids [9,10] will be promising as affinity ligands for specific base recognition by HPAC and AGE.

The concept of base recognition by the analogues is based on the idea that analogues having

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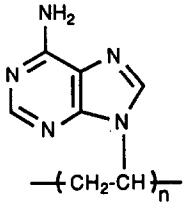
bases will interact with nucleic acids via complementary hydrogen bonding and/or stacking interaction, which will result in retardation of the mobility of nucleic acids having complementary bases. We selected poly(9-vinyladenine) (PVAd) as one of the fully synthetic vinyl polymer analogues of nucleic acids because it possesses advantages of ease of preparation, high solubility in aqueous media and stability against chemical- and enzyme-catalysed hydrolysis over natural polynucleotides and additionally in electrophoresis PVAd does not undergo electroosmotic flow because of its neutral character. Moreover, PVAd forms a complex with poly(U) by complementary hydrogen bonding, as established by UV and NMR spectroscopy [11,12].



PVAd

We have demonstrated the validity of PVAd as an affinity ligand for the nucleobase-selective separation of nucleic acids in HPAC [13,14], AGE [15] and capillary affinity gel electrophoresis (CAGE) [16–20]. PVAd was chemically bonded to silica gel to separate oligonucleotides differing in length from mixtures of oligoadenylic and oligouridylic acids with base selectivity. Agarose–PVAd conjugated gel separated poly(adenylic acid) [poly(A)] and poly(U) of similar size in affinity gel electrophoresis. Further, we have developed a capillary filled with polyacrylamide–PVAd conjugated gel, which selectively separated oligothymidylic acids from mixtures of oligothymidylic and oligodeoxyadenylic acids with high resolution and high speed. The effects of urea, temperature and molecular mass and concentration of PVAd in a gel filled with polyacrylamide were examined to develop high-sensitivity and high-speed base recognition of oligonucleotides.

In this paper, a more detailed investigation and the efficiency of PVAd as an affinity ligand in AGE are described. This approach will also serve to elucidate the interaction of PVAd with nucleic acids which may not be detectable by usual spectroscopic methods.

EXPERIMENTAL

Materials

Boric acid, ethylenediaminetetraacetic acid (EDTA), bromophenol blue, xylene cyanol, ethidium bromide and methylene blue were of analytical-reagent grade from Nacalai Tesque (Kyoto, Japan). Tris(hydroxymethyl)aminomethane (Tris), agarose and ΦX174 Hae III and λ Hind III restriction enzyme fragments were obtained from Sigma (St. Louis, MO, USA), Takara (Kyoto, Japan) and Toyobo (Osaka, Japan), respectively. Poly(guanylic acid) [poly(G)] was purchased from Pharmacia–LKB (Uppsala, Sweden). Poly(A), poly(inosinic acid) [poly(I)], poly(cytidylic acid) [poly(C)], triple-stranded [poly(A)–poly(U)–poly(U)], double-stranded [poly(deoxyadenylic acid)–poly(deoxythymidylic acid)] [poly(dA)–poly(dT)], [poly(dA–dT)–poly(dA–dT)], [poly(dG–dC)–poly(dG–dC)], single-stranded M13 mp8 phage DNA and calf thymus DNA were purchased from Sigma. pUC18 plasmid DNA was a gift of Professor Higashi and Dr. Uchiumi of the Department of Biology, Kagoshima University.

PVAd was prepared by the polymerization of vinyladenine according to the literature [16,21]. The PVAd obtained was fractionated using an ultrafiltration technique (Amicon 8200 standard cell) with Amicon membranes (molecular mass cut-offs 10 000, 30 000 and 50 000) under nitrogen pressure (2.0 kg/cm^2). Three PVAd samples having molecular mass ranges of <10 000, 10 000–30 000 and 30 000–50 000 were obtained.

Sonication

As most commercially available nucleic acids have high molecular masses and a very broad range of distribution of molecular mass and are not suitable for affinity gel electrophoresis, they were sonicated in TE buffer (10 m*M* Tris–1 m*M* EDTA, pH 7.5) containing 0.5 *M* NaCl. Sonication was conducted under nitrogen or helium at 0–8°C using a Tomy Seiko sonicator (Model

UR-200P) with a high gain and a 25% pulse cycle using a programmable timer (Kagaku Kyoeisha, Osaka, Japan) to prevent heating. After sonication, the solution was filtered through a 0.45-μm Millipore filter and then dialysed in a solution of 0.1 *M* NaCl and 1 m*M* EDTA using a Spectra Por membrane (molecular mass cut off 6000–8000) that had been pretreated twice with a boiling solution of EDTA (2 m*M*) for 15 min. The dialysed solution was lyophilized and the sample obtained was stored at −20°C until used. The relative size of the sonicated nucleic acid was estimated against λ Hind III restriction enzyme fragments by agarose (0.7%) gel electrophoresis. The sizes of the sonicated nucleic acids were in the range 200–400 base pairs (bp) except for poly(A) (300–700 bp). Double-stranded poly(A)–poly(U), poly(G)–poly(C) and poly(I)–poly(C) were prepared by mixing equimolar amounts of the sonicated single-stranded polynucleotides.

Gel electrophoresis

Gel electrophoresis was performed according to a protocol [22] using a submarine-type gel apparatus (Bio-Rad DNA SUB CELL) at a constant voltage (100 V) on an ATTO AE-8350 power supply usually for 2 h using TBE buffer (89 m*M* Tris–89 m*M* boric acid–2 m*M* EDTA, pH 8). Agarose gel (0.7 or 1.4%) was prepared in the usual manner using a Mupid Gel Maker Set (Cosmo Bio, Tokyo, Japan) and agarose-PVAd conjugated gel was prepared by dissolving agarose and the desired amount of PVAd in buffer. Nucleic acids were dissolved in TE buffer (10 m*M* tris–1 m*M* EDTA) and the solution (10–20 m*M*) was mixed with glycerol containing bromophenol and xylene cyanol. Denaturation of nucleic acids was done by heating the solution of nucleic acids to 95 or 100°C, followed by cooling quickly to 0°C.

After electrophoresis, the gels were placed on a TLC sheet (20 × 20 cm) (Merck, Art. 5735) containing fluorescence agents, and the positions of the nucleic acids loaded were recorded under UV light. The gels were first stained with the intercalating dye ethidium bromide to reveal DNA and double-stranded RNA by fluorescence under UV light (312 nm; Cosmo-Bio, CSF-20B transilluminator). Because ethidium bromide was not satisfactory as a stain for a single-stranded RNA, the gels were rinsed for 15 min in 1.0 *M* acetic acid to lower the pH of the gels and were then stained with 0.2 wt.% methylene blue dissolved in an acetate buffer for 1–2 h. The excess stain was removed from the gels using a continuous flow of fresh water for about 12 h. The blue bands of single-stranded RNA in the gel were clearly observed. It has been reported that methylene blue had a high affinity for binding to RNA, but the binding of ethidium bromide to poly(A) and poly(U) was negligibly small [23]. The electrophoresis experiments were repeated several times and the reproducibility was satisfactory.

Photographs were taken with a Polaroid ACMEL CRT camera (M-0851) with Polaroid Type 665 P/N film.

Polyacrylamide gel (5%) (acrylamide: bisacrylamide = 19:1) was prepared in a similar manner to agarose gel under a nitrogen atmosphere.

Measurements

UV spectra and "melting temperature" (T_m) corresponding to a 50% transition of complexes were obtained with a Hitachi Model 200-20 spectrophotometer in a 1.0-cm path-length quartz cell equipped with a temperature controller (Tanson TC 3). Hypochromicity was obtained by continuous variation mixing curves in 0.1 *M* phosphate buffer (pH 7.0) containing 50 m*M* NaCl at 25°C by monitoring the absorbance at 253 and 290 nm for PVAd or the poly(A) and poly(I) systems. Melting curves were obtained by monitoring the absorbance change at 253 and 290 nm with slow heating at a rate below 0.5°C/min.

RESULTS AND DISCUSSION

Electrophoresis of DNA

We first examined the effects of molecular mass and concentration of PVAd on the mobility of double-stranded DNA in agarose gel (0.7%) electrophoresis. It should be noted that the elution of PVAd from the gel is negligible. The double-stranded DNA interacts weakly with

PVAd as determined by UV spectroscopic analysis. The hyperchromicity of calf thymus DNA to PVAd was about 5% based on the continuous variation mixing curve. Therefore, double-stranded DNA was suitable as a standard to calibrate the mobilities of other nucleic acids in AGE containing PVAd. Three PVAds having molecular mass ranges of <10 000, 10 000–30 000 and 30 000–50 000 and a vinyladenine monomer were used as affinity ligands. The numbers of adenine bases of these PVAd were <60, 60–180 and 180–310, respectively.

The results of the mobilities of DNAs including covalently closed circular (ccc) and open circular (oc) pUC 18 plasmid in agarose gel electrophoresis in the absence and presence of different PVAds with respect to molecular mass and concentration are illustrated in Fig. 1.

Fig. 1a demonstrates that the mobilities of linear and circular DNAs decreased as the molecular mass of PVAd increased. The retardation of DNA mobility was not much different when the molecular mass of PVAd ranged from 10 000 to 50 000. In these electrophoreses, the concentration of PVAd was held constant at 10% (w/w) relative to agarose. The concentration of PVAd also affects the mobility of DNA. An increase in the concentration of PVAd resulted in a decrease in the migration of DNA, but the retardation was almost constant at more than 10% (w/w) PVAd. Although the interaction between equimolar PVAd and double-stranded DNA was weak based on UV spectroscopy, excess of PVAd entrapped within the gel matrix will cause retardation of the mobility in electrophoresis. Consequently, we employed PVAd having a molecular mass of >30 000 for affinity gel electrophoresis, and the concentration was held constant at 10% (w/w) relative to agarose throughout.

A dramatic decrease in mobility of DNA was found when the double-stranded DNA was denatured by heating at 95°C for 3 min (Fig. 2, lane 2). We thought that this was due to hydrogen bonding or base pairing of PVAd with the denatured, probably, single-stranded DNA. In order to elucidate this, the single-stranded (+)-M13mp8 was loaded on to the same gel. As shown in lanes 3 and 4, the DNA was almost completely trapped at the upper part of the gel by PVAd. This may be applicable to selective

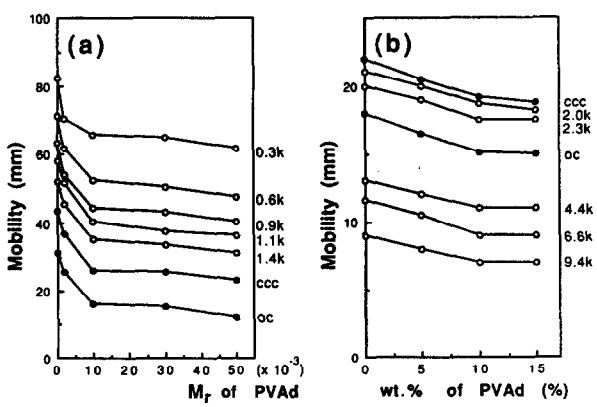


Fig. 1. (a) Effect of the molecular mass of PVAd on the mobility of ΦX174 Hae III fragments and (b) effect of the concentration of PVAd on the mobility of λ Hind III fragments. (a) The gel contained 0.7% agarose and 10% (w/w) PVAd and (b) the molecular mass of PVAd in the gel was 30 000–50 000. Gel electrophoresis was performed using TBE buffer (89 m*M* Tris–89 m*M* boric acid–2 m*M* EDTA, pH 8). The abbreviations ccc, oc and k mean covalently closed circular, open circular and 10^3 (base pairs), respectively.

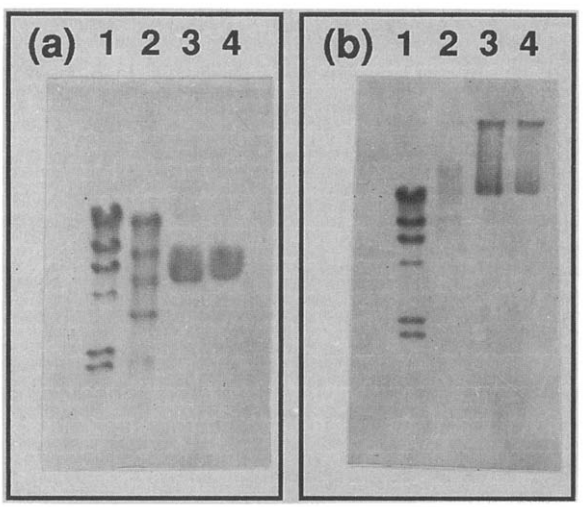


Fig. 2. Electrophoresis of DNA on (a) agarose gel (0.7%) and (b) agarose–PVAd gel. The concentration and molecular mass of PVAd in the gel were 10% (w/w) and 30 000–50 000, respectively. Lanes: 1 = Hind III restriction enzyme digests of λ DNA; 2 = sample 1 heated at 95°C for 3 min; 3 = single-stranded M13 mp8 phage DNA; 4 = sample 3 heated at 95°C for 3 min. Gel electrophoresis was performed using TBE buffer (89 m*M* Tris–89 m*M* boric acid–2 m*M* EDTA, pH 8). The gels were stained with ethidium bromide.

separation of double-stranded DNA in mixtures of single- and double-stranded DNAs [24].

The ability of PVAd for specific base recognition of DNA was examined using several DNAs with different base sequences and dT content such as poly(dA–dT)$_2$, poly(dG–dC)$_2$, calf thymus DNA (dT content *ca.* 30%) and poly(dA)–poly(dT) in agarose–PVAd conjugated gel electrophoresis (Fig. 3, lanes 1–13, and Fig. 4, lanes 1 and 2). All double-stranded DNAs were denatured by heating them at 95 or 100°C to give single-stranded DNAs. It was found that only the denatured calf thymus DNA was trapped by PVAd (lanes 7 and 9 in Fig. 3), and other DNAs migrated rapidly in both the gels. We first expected that the single-stranded poly(dA–dT) and poly(dT) may be adsorbed most strongly in the agarose–PVAd conjugated gel owing to complementary hydrogen bond formation between dT bases of poly(dA–dT) and poly(dT) and adenyl moieties of PVAd. However, the differences in the mobility of the denatured poly(dA–dT)$_2$ and poly(dA)–poly(dT) with and without PVAd were not regarded as significant. This may be due to rapid intramolecular renaturation of the resulting single-stranded poly(dA–dT) and intermolecular renaturation of the poly(dA) and poly(dT). In the former instance, when a sequence of bases is a complementary sequence in the same chain as in poly(dA–dT), the chain will fold back to form a duplex hairpin by base pairing between complementary sequences. Therefore, the poly(dA–dT) having a duplex hairpin, which was expected to retain its original half size, migrated faster than the poly(dA–dT)$_2$ without interacting with PVAd. In the latter instance, the poly(dT) will renature with the poly(dA) before it interacts with the immobilized PVAd in the gel, and then the resulting double-stranded poly(dA)–poly(dT) may migrate like the untreated substance. The interaction of poly(dT) with PVAd might be strong because the migration of oligo(dT)$_{12-18}$ in a capillary filled with polyacrylamide gel containing only 0.05% (w/w) PVAd was strongly retarded by PVAd even in the presence of an excess of urea such as 7 *M*, as reported previously [16].

The single-stranded calf thymus DNA, of which the base sequence may not be regular enough to form a stable duplex hairpin, easily interacted with PVAd by base pairing to result in electrophoretic retardation.

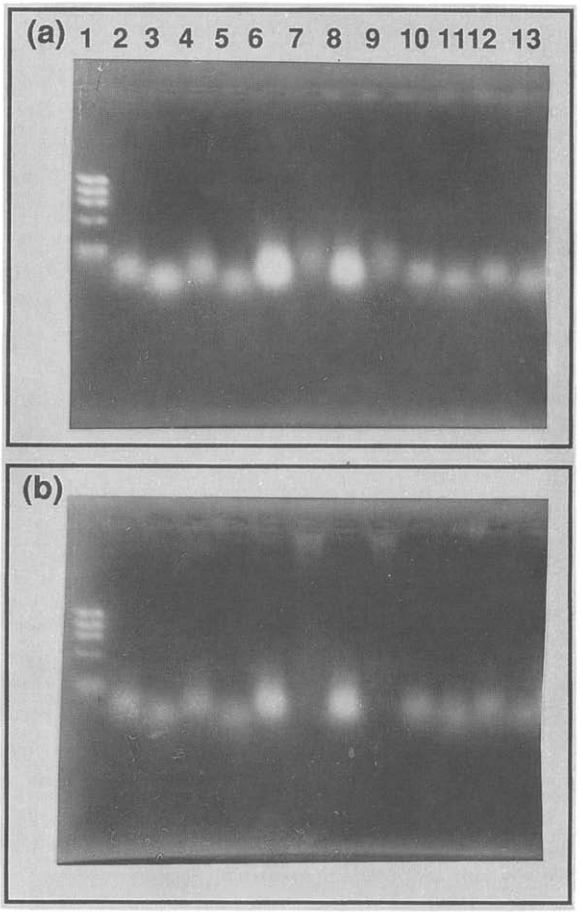


Fig. 3. Electrophoresis of DNA on (a) agarose gel and (b) agarose–PVAd gel. Conditions as in Fig. 2. Lanes: 1 = Hae III restriction enzyme digests of ΦX174 DNA; 2 = poly(dG–dC)$_2$; 3 = sample 2 heated at 100°C for 10 min; 4 = poly(dG–dC)$_2$–PVAd [1:1 (mol/mol)]; 5 = sample 4 heated at 100°C for 10 min; 6 = calf thymus DNA; 7 = sample 6 heated at 100°C for 10 min; 8 = calf thymus DNA–PVAd [1:1 (mol/mol)]; 9 = sample 8 heated at 100°C for 10 min; 10 = poly(dA–dT)$_2$; 11 = sample 10 heated at 100°C for 10 min; 12 = poly(dA–dT)$_2$-PVAd [1:1 (mol/mol)]; 13 = sample 12 heated at 100°C for 10 min. The gels were stained with ethidium bromide.

Electrophoresis of polynucleotides

Based on imino ^{1}H and ^{31}P NMR, UV and circular dichroism spectroscopy, PVAd was found to form a complex with poly(U) by com-

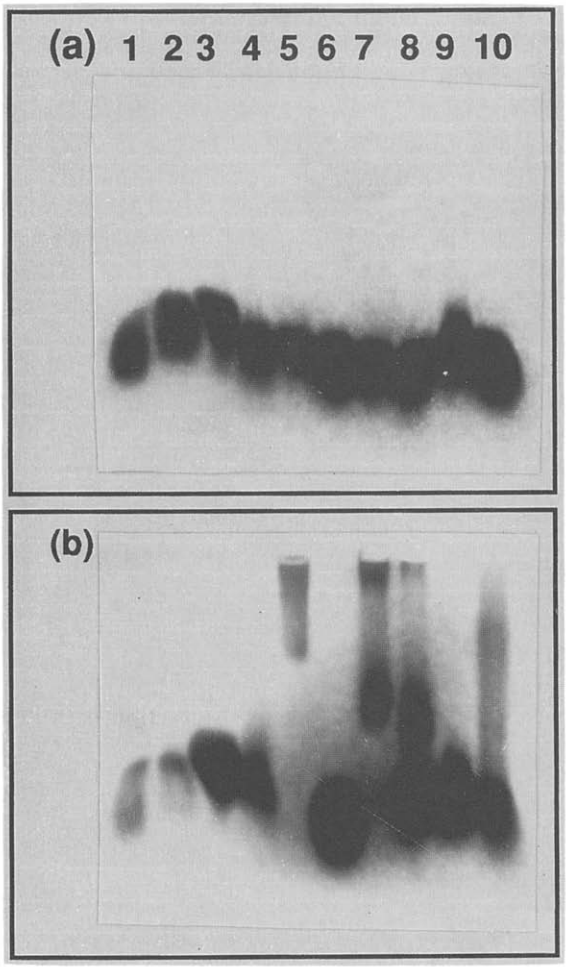


Fig. 4. Electrophoresis of DNA and polynucleotides on (a) agarose gel and (b) agarose–PVAd gel. Conditions as in Fig. 2. Lanes: 1 = poly(dA)–poly(dT); 2 = sample 2 heated at 95°C for 3 min; 3 = poly(A); 4 = poly(G); 5 = poly(I); 6 = poly(C); 7 = poly(U); 8 = poly(A)–poly(U); 9 = poly(G)–poly(C); 10 = poly(I)–poly(C). The gels were stained with methylene blue.

plementary hydrogen bonding [12]. The complex formation was also established in agarose–PVAd conjugated gel electrophoresis [15,25]. The mobility of poly(U) was considerably retarded by PVAd, but that of poly(A) was not significantly affected by PVAd. Such base recognition ability of PVAd can be utilized for the base-specific separation of oligonucleotides and oligodeoxynucleotides in HPLC [14] and in capillary gel electrophoresis [16–20]. Here, we employed five polynucleotides having different bases to investigate the more detailed base-specific recognition ability of PVAd in agarose–PVAd conjugated gel. The results serve to elucidate the characteristics of complex formation of PVAd with nucleic acids, which may not easily be detectable by the usual spectroscopic methods.

Fig. 4 shows the electrophoretic patterns of single- and double-stranded polynucleotides on agarose and agarose–PVAd conjugated gels. Among the five single-stranded polynucleotides with different base composition, poly(U) and poly(I) were base-specifically recognized by PVAd (lanes 5 and 7) and those electrophoretic mobilities were significantly diminished, but other polynucleotides exhibited almost the same electrophoretic mobility in both gels. Base pairing between poly(U) and PVAd, which is established by NMR and UV spectroscopy, will lead to the retardation of migration of poly(U). The situation seems to be very similar with poly(I), as it forms a double helix with poly(A) by hydrogen bonding between the bases. Similarly, PVAd will be expected to interact with poly(I). However, no clear spectroscopic variations in base pairing between PVAd and poly(I) were observed in UV spectroscopy. Hypochromicity and melting between them were not regarded as significant, whereas those for a mixture of poly(A) and poly(I) were clearly observed with *ca.* 25% of hypochromicity, *ca.* 60% of hyperchromicity and a T_m of 33°C. The exact nature of the interaction between PVAd and poly(I) cannot be explained. Weak hydrogen bonding interactions or hydrophobic interactions between them, which will be small enough to escape detection by UV spectroscopy, should be considered.

Lanes 8, 9 and 10 in Fig. 4 demonstrate that the duplexes of poly(A)–poly(U) and poly(I)–poly(C) are resolved into two bands even without heat treatment (b), but the bands are single in agarose gel (a). Band broadening occurred especially with poly(I)–poly(C), probably owing to strong binding to the immobilized PVAd as shown in Fig. 4b. Poly(G)–poly(C) migrated similarly in both gels. These results suggest that the immobilized PVAd interacted with the duplexes of poly(A)–poly(U) and poly(I)–poly(C), then pulled off the poly(U) and poly(I) strands

from the duplexes and complexed with them to give the PVAd–poly(U) and PVAd–poly(I) complexes, respectively. Therefore, the upper broad bands may be assigned to the PVAd–poly(U) and PVAd–poly(I) complexes and the lower sharp bands to the free poly(A) and poly(C), respectively. The base-pairing ability of PVAd to poly(U) and poly(I) seems to be superior to that of poly(A) under these conditions.

Next, in order to investigate the interaction between PVAd and multi-stranded polynucleotides, double- and triple-stranded polynucleotides were loaded and the gels were stained with ethidium bromide and methylene blue.

Fig. 5 shows the electrophoretic patterns of double- and triple-stranded polynucleotides and/or DNA in agarose gel with (b and d) and without (a and c) PVAd. The patterns observed in Fig. 5b and d were complicated. When ethidium bromide was used as a stain, only single bands appeared at around 1.4 kbp of DNA size marker (Fig. 5b, lanes 4–7). On the other hand, staining with methylene blue gave additional bands smaller in size than the former bands (Fig. 5d). Ethidium bromide can stain multi-stranded nucleic acids but is not suitable for single-stranded nucleic acids. Methylene blue can stain both single- and multi-stranded nucleic acids. Therefore, the lower bands, stained only with methylene blue should be the free poly(A) and the upper bands, stained with two dyes, will be the PVAd–poly(U) complex, to which ethidium bromide will intercalate and fluoresce. The strong interaction of PVAd with the Hoogstein-paired poly(U) of the triple-stranded poly(A)–2poly(U) was established by UV and ^{31}P NMR spectroscopy [12]. However, a significant interaction between PVAd and the Watson–Crick-paired poly(U) could not be detected by these methods. Therefore, a weak interaction between PVAd and the Watson–Crick-paired poly(U) of the double-stranded poly(A)–poly(U) may be amplified throughout the affinity gel electrophoresis, which will cause retardation of the mobility of the poly(U).

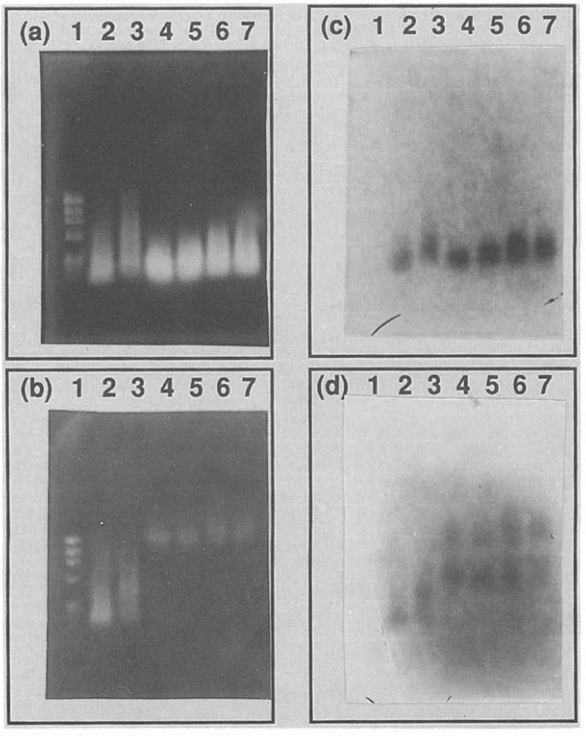


Fig. 5. Electrophoresis of DNA and polynucleotides on (a) agarose gel and (b) agarose–PVAd gel. Conditions as in Fig. 2. The gels were stained with (a and b) ethidium bromide and (c and d) methylene blue. Lanes: 1 = Hae III restriction enzyme digests of ΦX174 DNA; 2 = poly(dA)–poly(dT); 3 = sample 2 heated at 95°C for 3 min; 4 = poly(A)–poly(U); 5 = sample 4 heated at 95°C for 3 min; 6 = poly(A)–2poly(U); 7 = sample 6 heated at 95°C for 3 min.

In conclusion, PVAd was found to be effective as an affinity ligand in affinity gel electrophoresis. PVAd specifically recognizes not only poly(U) but also poly(I), probably by hydrogen bond formation, and does not interact with poly(A), poly(G) and poly(C) in agarose gel. PVAd interacts only with a single-stranded DNA, the sequence of which is not regular enough to form a stable duplex hairpin structure. As many synthetic nucleic acid analogous have been prepared [10], most of them should be utilized as affinity ligands in affinity gel electrophoresis, and our approach developed here will be valuable for the base and shape recognition of nucleic acids.

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