Title	Biological activities of synthetic polymers(Dissertation_　　　)
Author(s)	Sawa, Tomohiro
Citation	Kyoto University (　　　　)
Issue Date	1996-07-23
URL	http://dx.doi.org/10.11501/3117178
Right	
Type	Thesis or Dissertation
Textversion	author

BIOLOGICAL ACTIVITIES OF
SYNTHETIC POLYMERS

Tomohiro Sawa

1996

Contents

ABBREVIATIONS

AGE	affinity gel electrophoresis
CAGE	capillary affinity gel electrophoresis
CF	5(6)-carboxyfluorescein
CGE	capillary gel electrophoresis
dA	deoxyadenylic acid
dT	thymidylic acid
DMEM	Dulbecco's modified Eagle medium
FBS	fetal bovine serum
MA-AU	poly(maleic acid-alt-allylurea)
MA-BD	poly(maleic acid-alt-2-buten-1,4-diol)
MA-CDA	poly(maleic acid-alt-7,12-dioxaspiro[5.6]dodec-9-ene)
MA-MP	poly(maleic acid-alt-4-methyl-4-penten-2-one)
MA-ST	poly(maleic acid-alt-styrene)
MEM	Eagle's minimal essential medium
PBS	phosphate buffered saline
PVAd	poly(9-vinyladenine)

INTRODUCTION

Synthetic polymers have several advantages over naturally occurring polymers. First of all, virtually unlimited numbers of structures are possible for synthetic polymers. One can design a wide variety of polymer structures, which may not even exist in nature.[1]

By designing, properties of a synthetic polymer including interaction of a polymer with cell components can be controlled.[2] For example, a synthetic polymer can have superior mechanical strength compared to naturally occurring polymers. Chemical properties of a synthetic polymer can also be arranged. For instance, a polymer may be designed to be either far more durable to an enzymatic attack than naturally occurring polymers or, in opposite, to be biodegradable.

Various synthetic polymers interact with cell or cell components such as DNA, RNA,[3] and enzymes,[4] and many of those polymers show interesting biological activities. For example, poly(ethylenimine), poly(acrylic acid), poly(methacrylic acid), and copovithane show antitumor activities, and their antitumor activities have extensively been studied because of therapeutic interest.[5]

In order to design a synthetic polymer that can interact with cell components, two strategies are mainly possible. One strategy is to adopt a part or whole functional structure in a known biologically active substance of natural origin into the backbone structure of a synthetic polymer which has other desirable properties. For instance, insulin, a growth factor, was immobilized on a non-biodegradable polymer

1

membrane. The membrane accelerats cell growth and is useful as cell culture matrices.[6] Kobayashi *et al.* reported that lactose bearing polystyrene enhances the adhesion of hepatocytes to a cell culture dish.[7] This enhanced adhesion of hepatocytes is thought to be due to the binding between the lactose residues of the polymer and lactose receptors on hepatocytes.[7] This "hybrid" approach is straight-forward, and the properties of a resulting polymer can be often predictable. On the other hand, the scope of this approach is limited by availability of parent structures.

Another strategy focuses on the relation between a biological activity and physicochemical characteristics of a polymer. In the past, anionic polysulfonates and polyphosphates of either natural or synthetic origin was known to be potent inhibitors of transplanted tumors in mice.[8, 9] Based on a hypothesis that this inhibition of tumor growth may conceivably be a function of the density and distribution of anionic charges within those polyelectrolyte molecules, Regelson and his coworkers synthesized and examined synthetic polymers of different charge densities for the tumor-inhibiting property and found that fully synthetic polymers such as ethylene-maleic acid copolymer could show significant antitumor activity.[10] In their study, the charge density of the polymer was controlled by amidation of the carboxyl groups on the copolymer. The maximum inhibition of tumor by the copolymer was observed at a certain optimal ratio of the carboxylate anion and the amide. Neither the polymer that was completely amidated nor the monomeric species showed the antitumor activity.[10]

Regelson's work showed a possibility of a new approach to obtain a biologically active synthetic polymer. One does not necessarily have to mimic a structure of a known biologically active substance. Instead, one may design a polymer that has certain physicochemical properties which is required for a particular biological effect. This approach allows us to design bioactive synthetic polymers more flexibly. Here, however, one has to know in advance what kind of physicochemical characteristics is required to obtain a polymer with a desired biological activity.

This thesis consists of two parts. In Part I, the interaction between a synthetic polymer poly(9-vinyladenine) (PVAd) and nucleotides was investigated using a newly developed technique, capillary affinity gel electrophoresis (CAGE). In part II, this thesis describes a novel growth promotion activity found for a synthetic polyanionic polymer poly(maleic acid-alt-7,12-dioxaspiro[5.6]dodec-9-ene) (MA-CDA) and interaction of MA-CDA with cell plasma membranes.

REFERENCES

[1] Donaruma, L. G. (1975) *Prog. Polym. Sci.*, 4, 1-25.

[2] Jozefonvicz, J. and Jozefowicz, M. (1990) *J. Biomater. Sci. Polymer Edn*, 1, 147-165.

[3] Pitha, J. (1977) *Polymer*, 18, 425-430.

[4] Holler, E., Achhammer, G., Angerer, B., Gantz, B., Hambach, C.,

Reisner, H., Seidel, B., Weber, C., Windisch, C., Braud, C.,
Guerin, P. and Vert, M. (1992) *Eur. J. Biochem.*, 206, 1-6.

[5] Seymour, L. (1991) *J. Bioact. Compt. Polym.*, 6, 178-216.

[6] Ito, Y., Liu, S. Q., Orihara, T. and Imanishi, Y. (1994) *J. Bioact. Compt. Polym.*, 9, 170-183.

[7] Kobayashi, A. and Akaike, T. (1986) *Makromol. Chem., Rapid Commun.*, 7, 645-650.

[8] Regelson, W. and Holland, J. F. (1958) *Nature*, 181, 46.

[9] Muehlbarchar, C., Straumfjord, J., Jr., Hummel, J. P. and Regelson, W. (1959) *Canser Res.*, 19, 907.

[10] Regelson, W., Kuhar, S., Tunis, M., Fields, J., Johnson, J. and Gluesenkamp, E. (1960) *Nature*, 186, 778-780.

Part I

INTERACTION BETWEEN NUCLEIC ACIDS AND ITS VINYLANALOGUE

INTRODUCTION

One strategy to design a synthetic polymer that can interact with cell components is to introduce a part or whole structure of a naturally occurring biologically active molecule into the polymer. With expectation of a synthetic polymer that interacts with DNAs, varieties of synthetic polymers bearing nucleobases at the side chains of the polymer were synthesized, and their biological activities, especially complexation with naturally occurring nucleic acids, were investigated.[1] These polymers are often referred as nucleic acid analogues.

Figure 1 shows the chemical structures of some typical nucleic acid analogues. Poly(vinyl) *1*,[1] poly(acrylate) and poly(methacrylate) *2*,[1] and poly(acrylamide) and poly(methacrylamide) *3*,[1] analogues can be synthesized by free radical polymerization of corresponding comonomers. A poly(ethylenimine) analogue *4*[1] is prepared by grafting of nucleobase derivatives onto poly(ethylenimine) backbones. A peptide nucleic acid analogue *5* is obtained using Merrifield solid phase synthesis.[2]

Interaction of those analogues with nucleic acids was certainly observed. Poly(ethylenimine) analogues formed complexes with polynucleotides.[1] Peptide nucleic acid type analogues interacted with DNA oligomers those have complementary bases.[2]

These nucleic acid analogues have been used also in studies of biological activities of nucleic acids. For instance, in a study of
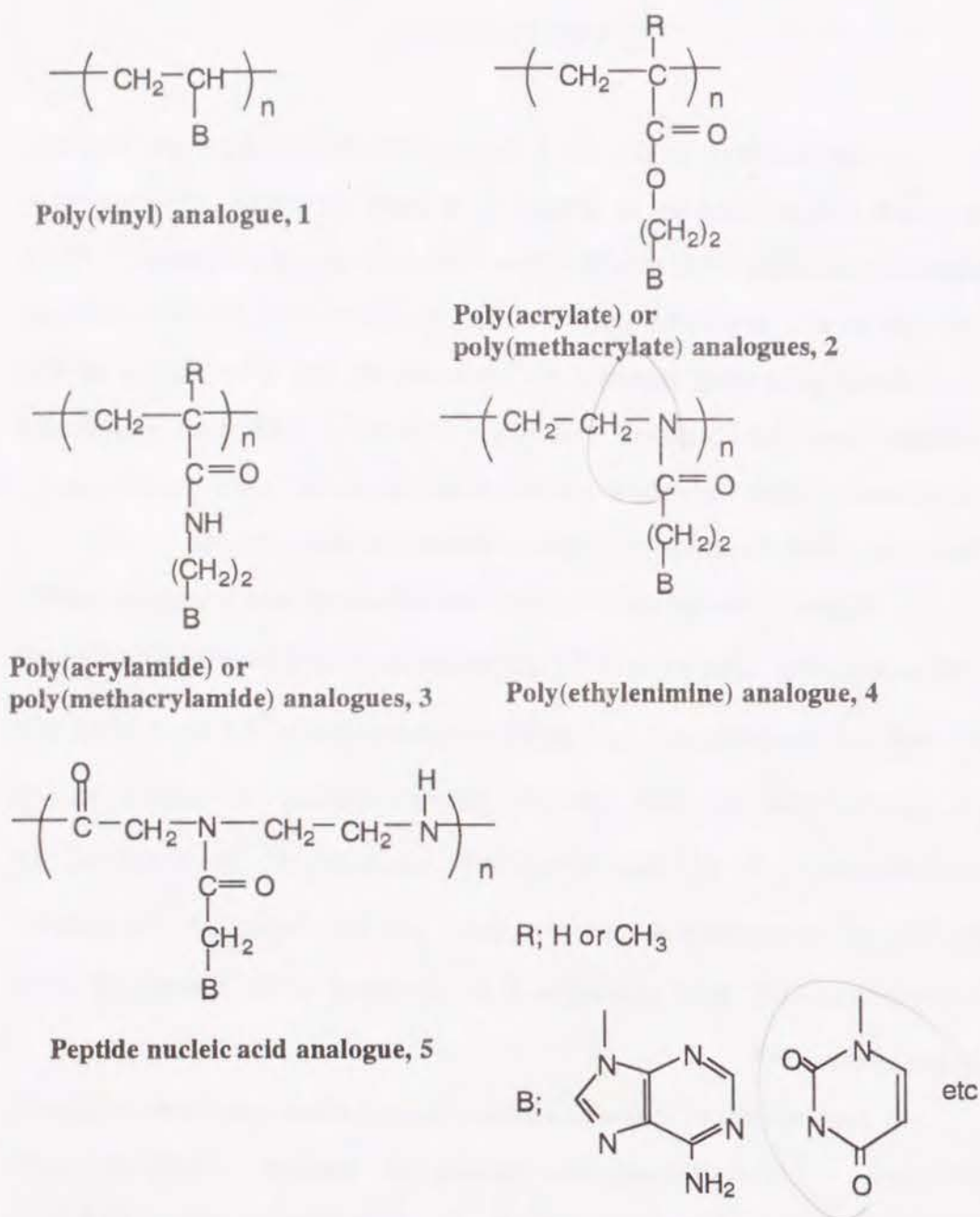
$$\left(CH_2-\underset{\underset{B}{|}}{CH}\right)_n$$

Poly(vinyl) analogue, 1

$$\left(CH_2-\underset{\underset{\underset{\underset{B}{|}}{(CH_2)_2}}{\underset{|}{O}}}{\overset{\overset{R}{|}}{\underset{|}{C}}}\right)_n$$

Poly(acrylate) or poly(methacrylate) analogues, 2

$$\left(CH_2-\underset{\underset{\underset{\underset{B}{|}}{(CH_2)_2}}{\underset{|}{NH}}}{\overset{\overset{R}{|}}{\underset{\underset{C=O}{|}}{C}}}\right)_n$$

Poly(acrylamide) or poly(methacrylamide) analogues, 3

$$\left(CH_2-CH_2-\underset{\underset{\underset{\underset{B}{|}}{(CH_2)_2}}{\underset{C=O}{|}}}{N}\right)_n$$

Poly(ethylenimine) analogue, 4

$$\left(\overset{\overset{O}{\|}}{C}-CH_2-\underset{\underset{\underset{\underset{B}{|}}{CH_2}}{\underset{C=O}{|}}}{N}-CH_2-CH_2-\overset{\overset{H}{|}}{N}\right)_n$$

Peptide nucleic acid analogue, 5

R; H or CH₃

B;

etc

Figure 1. Structures of typical nucleic acid analogues.

interferon induction activity of a complex of poly(inosinic acid) and poly(cytidylic acid), a poly(vinyl) analogue, poly(vinylcytosine), was used as a model nucleic acid in place of poly(cytidylic acid).[3]

Poly(9-vinyladenine) (PVAd) is one of those nucleic acid analogues, which carries adenine moieties on a poly(vinyl) backbone (Figure 1).[4-6] PVAd possesses several advantages over other nucleic acid analogues and natural polynucleotides. PVAd can be simply synthesized by polymerization of 9-vinyladenine.[1] The monomer also can be easily obtained. The poly(vinyl) backbone is inert to most of chemical reactions, and therefore PVAd has far higher stabilities against chemical or enzymatic hydrolysis compared with natural poly-nucleotides.[3]

Among nucleic acid analogues, PVAd shows one of the strongest interaction with single stranded DNAs and RNAs. As expected from the presence of adenine bases in its structure, PVAd formed a complex with poly(uridylic acid) (poly(U)) in an aqueous medium.[4,6] Spectroscopic investigations including UV, CD, and ^{1}H- and ^{31}P-NMR studies suggest the complementary hydrogen bonding between the adenine moieties of PVAd and uracil bases of poly(U) for the complex formation.[7] In addition PVAd could form a complex also with naturally occurring RNA and DNA.[8,9] Although the formation of complementary hydrogen bonding between the adenine moiety of PVAd and uracil or thymine base of nucleic acid in the complex was speculated,[8] the detail of the complexation was yet to be elucidated.

PVAd showed inhibition of DNA replication, RNA

transcription, reverse transcription, or protein synthesis in a cell free system.[3] These inhibition effects were observed only for a template DNA or RNA that could form a complex with PVAd.[3] PVAd inhibited also the *in vitro* replication of Leukaemia virus.[3] In these activities, PVAd is thought to function in a similar manner as antisense nucleic acids by its complexation with nucleic acids.

The interaction between nucleic acid analogues and intact nucleic acids has been used for base specific separation of nucleic acids. Most often, nucleic acids are separated with slab gel electrophoresis (GE) based on a molecular sieving effect of gel. Introduction of ligands those show a specific interaction with nucleic acids adds an ability to GE an affinity promoting separation of nucleic acids. In the previous studies, PVAd was used as the ligand in affinity gel electrophoresis (AGE) separation of polynucleotides. Separation of poly(A) from poly(U) with similar molecular weight was achieved by the AGE system, though the separation of the two polynucleotides was difficult with conventional GE.[7,10] Naturally occurring RNAs or single stranded DNAs also was separated from double stranded DNAs, because the latter does not bind to PVAd.[7,10]

Also, AGE can be a valuable tool for quantitative study of specific interaction between an analyte and a ligand. Theoretical and practical aspects of AGE for determination of the apparent dissociation constant of a ligand-analyte complex are documented by Horejsi *et al.*[11,12]

Using an AGE system with PVAd physically immobilized into

a gel matrix, Yashima *et al.* demonstrated the validity of AGE as a method to determine the interaction between PVAd and nucleic acids.[7,10] The order of the binding strength of polynucleotides to PVAd was poly(I) > poly(U) > poly(G) ~ poly(A) >> poly(C) in AGE.[7,10]

In AGE, however, detection of nucleic acids separated is often time consuming and even difficult. For instance, in the AGE separation of DNAs using PVAd ligands, ethydium bromide was not suitable for the detection of DNA (fragment bands) because of poor binding of ethydium bromide to PVAd-DNA complexes. In such a case, DNA may have to be radioactively labeled in advance. In addition, for a more precise study of an analyte-ligand interaction, a higher resolution in AGE is desirable.

This weakness of AGE may be covered by combining AGE with capillary electrophoresis (CE). In CE, electrophoresis is carried out in a capillary, generally smaller than 100 μm in the diameter.[13] The resolution of electrophoretic separation is approximately proportional to the applying voltage. The much smaller size of a capillary allows more effective heat discharge than conventional electrophoresis. Therefore in CE, it is possible to apply a high voltage for high resolution in electrophoresis such as 200 V/cm.

Capillary gel electrophoresis (CGE) inherits these advantages of CE. Introduction of a gel matrix similar to that in slab gel electrophoresis into CE made it possible to separate oligo- and polynucleotides with higher resolution.[14] The resolution and speed of CGE are much better than those of slab gel electrophoresis. Typically

the theoretical plate number for the separation of polynucleotides is 1.5 ~ 3.0×10^7 plates/m in CGE, which is about 10 times larger than that in slab gel electrophoresis.[15] A typical run in CGE requires less than 30 min, which is much shorter than that in conventional slab gel electrophoresis. Using UV absorption, separated oligonucleotides can be detected in real time. In addition capillary can be reused multiple times. The amount of a sample necessary for a run can be very small, typically less than 10 ng per species.

However, this possibility to develop a new method capillary affinity gel electrophoresis (CAGE) by introducing the affinity mode to conventional CGE has never been extensively explored. Limited numbers of the previous attempts include chiral separation of tryptophan with albumin gel.[16] Also Guttman and Cooke expected specific separation of double-stranded DNA fragments by the intercalation of ethidium cations in CAGE. They used capillaries filled with linear polylacrylamide-dissolving ethidium bromide as the mobile ligand.[17] However, recognition of specific nucleic acid base in has never been achieved using their system.

The present study investigates a possibility of CAGE as a system for separation of oligonucleotides and analysis of the interaction between a ligand and DNA fragments. In Chapter 1, a CAGE system is constructed by immobilizing PVAd in a gel. Here a synthetic polymer PVAd is used as the first example of a gel immobilized macromonomer ligand (macroligand)[20,21] in CAGE. Previous experiences in AGE suggest that an electrically neutral

macroligand such as PVAd be suitable also for CAGE.

A naturally occurring DNA duplex consists of two complementary strands.[18] In most cases, an insertion of a mismatching base pair into the duplex decreases the thermodynamic stability of the duplex.[17-22] This destabilization is thought to be important for DNA repairing enzymes to detect and repair the mismatch.[17] The destabilization by a mismatching base pair is mainly explained by a decrease in the stacking interaction between the mismatching base pair and the neighboring base pairs.[17, 21, 22]

PVAd interacts with naturally occurring DNA and forms a complex.[7,10] The PVAd-DNA complex is expected to contain a lot of mismatching base pairs because the homogeneous adenine base sequence of PVAd would not exactly match the heterogeneous sequence of a native DNA. In theory, the effect of mismatching pairs on the thermodynamic stability of a PVAd-DNA complex should be reflected on the migration time of a DNA in the CAGE system.

In Chapter 2, the effect of a mismatching base in an oligonucleotide sequence on the interaction between PVAd and the oligonucleotide is actually evaluated using the CAGE system.

REFERENCES

[1] Takemoto, K. and Inaki, Y. (1981) *Adv. Polym. Sci.*, 41, 1-51.

[2] Egholm, M., Buchardt, O., Nielsen, P. E. and Berg, R. H. (1992) *J. Am. Chem. Soc.*, 114, 1895-1897.

[3]   Pitha, J. (1977) Polymer, 18, 425-430.

[4]   Pitha, J. and Ts'o, P. O. P. (1968) *J. Org. Chem.*, 33, 1341-1344.

[5]   Kondo, K., Iwasaki, H., Nakatani, K., Ueda, W., Takemoto, K. and Imoto, M. (1969) *Makromol. Chem.*, 125, 42.

[6]   Kaye, H. (1969) *J. Polym. Sci. Polym. Lett.*, 7, 1-5.

[7]   Yashima, E., Tajima, T., Miyauchi, N. and Akashi, M. (1992) *Biopolymers*, 32, 811-817.

[8]   Akashi, M., Iwasaki, H., Miyauchi, N., Sato, T., Sunamoto, J. and Takemoto, K. (1989) *J. Bioact. Compt. Polym.*, 4, 124-136.

[9]   Yashima, E., Suehiro, N., Miyauchi, N. and Akashi, M. (1993) *J. Chromatogr.*, 654, 151-158.

[10]   Yashima, E., Suehiro, N., Miyauchi, N. and Akashi, M. (1993) *J. Chromatogr.*, 654, 159-166.

[11]   Horejsi, V. and Ticha, M. (1986) *J. Chromatogr.*, 376, 49-67.

[12]   Horejsi, V. and Ticha, M. (1981) *J. Chromatogr.*, 216, 43-62.

[13]   Jorgenson, J. W. and Lukacs, K. D. (1981) *Anal. Chem*, 53, 1298-.

[14]   Baba, Y. and Tsuhako, M. (1992) *Trends Anal. Chem.*, 11, 280-287.

[15]   Baba, y., Matsuura, T., Wakamoto, K., Morita, Y., Nishitsu, Y. and Tsuhako, M. (1992) *Anal. Chem*, 64, 1221-1225.

[16]   Birnbaum, S. and Nilsson, S. (1992) *Anal. Chem*, 64, 2872-2874.

[17]   Guttman, A. and Cooke, N. (1991) *Anal. Chem*, 63, 2038-2042.

[18]   Watson, J. D. and Crick, F. H. C. (1953) *Nature*, 171, 737-738.

[19]   Tibanyenda, N., De Bruin, S. H., Haasnoot, C. A. G., Van der Marel, G. A., Van Boom, J. H. and Hilbers, C. W. (1984) *Eur. J. Biochem.*, 139, 19-27.

[20]   Aboul-ela, F., Koh, D. and Tinoco, J., I. (1985) *Nucleic Acids Res.*, 13, 4811-4824.

[21]   Werntges, H., Steger, G., Riesner, D. and Fritz, H.-J. (1986) *Nucleic Acids Res.*, 14, 3773-3790.

[22]   Ke, S.-H. and Wartell, R. M. (1993) *Nucleic Acids Res.*, 21, 5137-5143.

Chapter 1

Capillary affinity gel electrophoresis for a new method to investigate interaction between nucleic acids and its analogue

This chapter describes the construction of a capillary affinity gel electrophoresis (CAGE) system as a novel tool for both separation of oligodeoxynucleotides and evaluation of the interaction between an oligodeoxynucleotide and an immobilized affinity ligand. One of the most critical points in the construction of a CAGE system is preparation of a capillary that is filled with a gel incorporating an affinity ligand. There was no previous report that describes the immobilization of an affinity ligand into gel in the capillary.

In previous studies of affinity gel electrophoresis, three methodologies have been examined[1,2] to incorporate an affinity ligand into the gel matrix: (1) mobile ligands in gel matrix (Figure 1-1A), (2) macroligands entrapped within gel matrix (Figure 1-1B), and (3) ligands covalently bound to gel matrix (Figure 1-1C). The macroligand method has advantages over other two ligand incorporation methods.[1,2] Preparation of the gel matrix is much simpler than covalently bonded immobilization. Moreover, by immobilizing a ligand into gel matrix, one can assume the mobility of the ligand-analyte complex to be zero.[1,2] With this assumption, the migration time of the analyte can be simply

(A)   (B)   (C)
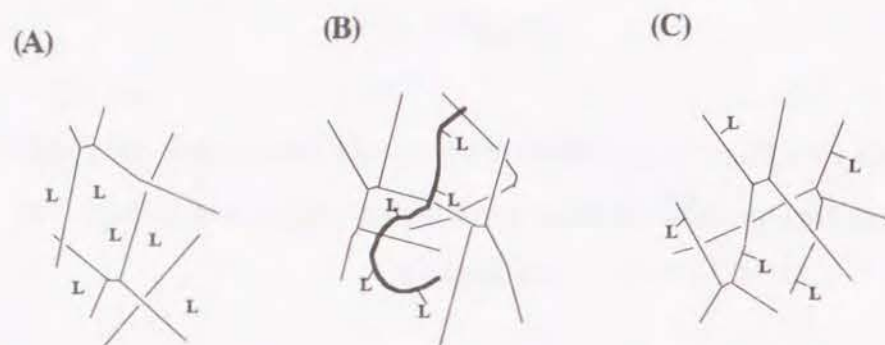
Figure 1-1. Schematic representations of affinity ligands in gel matrix; (A) mobile ligand method, (B) macroligand method, (C) directly immobilized ligand method .

related with the association constant of the ligand-analyte complex. With mobile ligand, the mobility cannot be negligible, and the mathematical expression of the behavior of the system could be considerably complex.

The CAGE system built in this study first adopted the macroligand approach. A nucleic acid analogue, poly(9-vinyladenine) (PVAd) was immobilized as a macroligand by *in situ* polymerization in a capillary. As the macroligand, PVAd was selected, because PVAd was satisfactorily used in the previous affinity gel electrophoresis (AGE) systems.[3,4] As already described, PVAd has other advantages such as strong binding to oligonucleotides. The CAGE system thus constructed is expected to possess advantage of both AGE and capillary gel electrophoresis (CGE). The reliability and the characteristic of CAGE system were examined in separation of model oligonucleotides.

MATERIALS AND METHODS

Materials

Acrylamide, N,N'-ethylenebis(acrylamide) (BIS), N,N,N',N'-tetramethylethylenediamine (TEMED), and ammonium peroxodisulfate were of the electrophoretic grade from Wako Pure Chemicals (Osaka). A mixture of oligodeoxyadenylic acids with chain length from 12 to 18, $(dA)_{12-18}$, and that of oligothymidylic acids, $(dT)_{12-18}$, were purchased from Pharmacia (Uppsala, Sweden). $(dT)_{15}$ was chemically synthesized using a Model 391 DNA synthesizer (ABI, Foster City, CA). Poly(9-vinyladenine) (PVAd) was prepared according to the literature[5] and fractionated using ultrafiltration with Amicon membranes (Amicon standard cell 8200, molecular weight cutoff 10,000, 30,000, and 50,000) under nitrogen pressure (2.0 kg/cm²). Polyimide-coated fused silica capillaries (375 µm outer and 100 µm inner diameters) were obtained from GL Sciences (Tokyo) and used in electrophoresis (effective length 22 cm and total length 42 cm). All other chemicals were obtained from Wako Pure Chemicals and used without further purification. The buffer used in the preparation of gel-filled capillaries and in the electrophoresis was a mixture of 0.1 M Tris and 0.1 M boric acid with an appropriate concentration of urea (pH 8.6).

Preparation of capillaries filled with PVAd-immobilized polyacrylamide gel

Capillaries filled with polyacrylamide gel were prepared

according to the literature.[6] The gel composition was fixed to be 8%T and 5%C. A similar method was used to produce capillaries filled with PVAd-immobilized polyacrylamide gel. To 5 ml of an aqueous mixture of PVAd, acrylamide, and BIS (8%T and 5%C) were added 20 μl of a 10 wt% aqueous solution of ammonium peroxodisulfate and 20 μl of a 10 vol% aqueous solution of TEMED. A capillary was filled with the resulting solution by using an apparatus described in Figure 1-2.
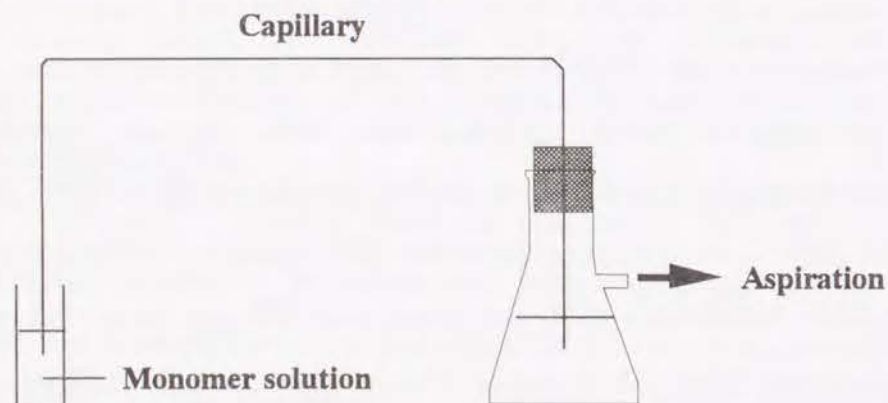
Figure 1-2. Schematic representation of the apparatus to introduce a monomer solution into a capillary.

Then the both ends of the capillary were sealed with a plastic film, and the capillary was warmed in a water bath (35 °C) for 5 h to complete polymerization of the monomers. The percentage of PVAd in a gel was obtained using equation 1.

$$\%PVAd = \frac{PVAd(g)}{acrylamide(g) + BIS(g) + PVAd(g)} \times 100 \qquad (1)$$

Capillary gel electrophoresis

Gel filled capillaries were mounted on a Model 270A capillary electrophoresis system (ABI) or a CE-800 capillary electrophoretic system (Jasco, Tokyo). There was no practical difference between the two systems that might affect the results. Electrophoresis was carried out by applying a voltage of 9 kV (214 V/cm). Before an actual measurement, a capillary filled with polyacrylamide gel was aged for 30 min to remove any unreacted monomer and initiator that might be remaining in the gel. Oligonucleotides (ca 1 unit/ml running buffer) were electrophoretically injected into the capillary by applying a voltage of 5 kV for 1 s. Oligodeoxynucleotides were detected using absorption at 260 nm. Electropherograms were processed on either a D-2500 integrator (Hitachi, Tokyo) or a CR-3A integrator (Shimadzu, Kyoto).

RESULTS

Capillary affinity gel electrophoresis of oligodeoxynucleotides using PVAd ligands

Figure 1-3 shows electropherograms of $(dA)_{12-18}$ under three different concentrations of immobilized PVAd. The separation of oligo(dA)s was not affected by the immobilization of PVAd in gel
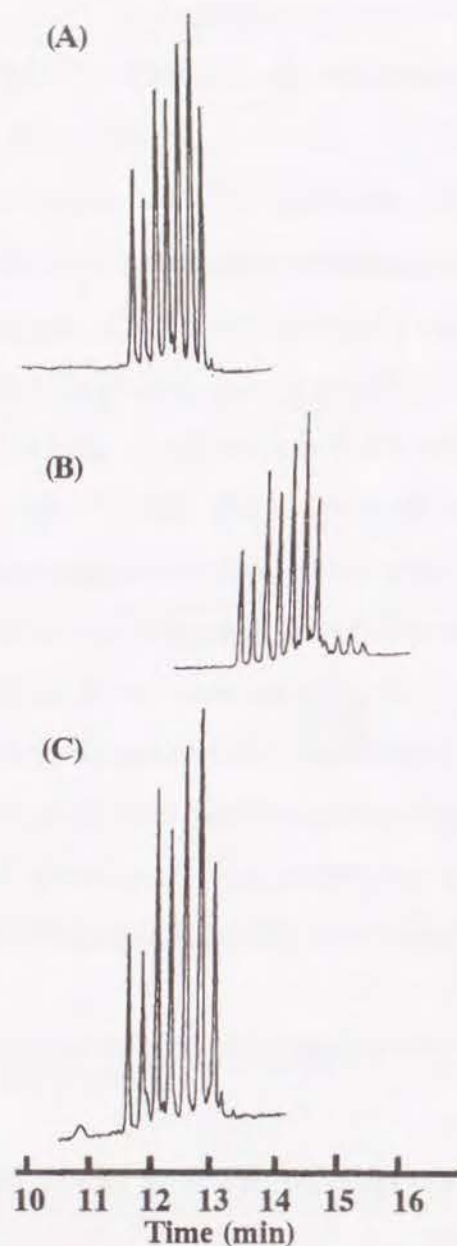
Figure 1-3. Separation of $(dA)_{12-18}$ in capillaries filled with PVAd-immobilized acryamide gel. Concentration of immobilized PVAd: (A) 0 %, (B) 0.05 %, (C) 0.2 %. Running buffer: 0.1 M Tris-borate and 5.6 M urea (pH=8.6). Capillary temperature: 30 °C.

matrix. Both in the absence and in the presence of PVAd, oligo(dA)s were baseline-resolved into seven bands within 15 min. The plate number calculated was $(1-8) \times 10^6$ plates/m.[7] Even in the presence of 0.2 % PVAd, there was no significant change of the peak shape compared with the case without PVAd.

The capillaries containing PVAd showed stability comparable to a capillary without PVAd. The average migration time of oligo(dA)$_{12}$ in ten repetitive measurements on a single capillary was 15.0 min with the relative standard deviation of 2.6% when the concentrations of PVAd and urea were 0.02 % and 4.0 M, respectively. In addition no change of the peak shape was observed during the ten measurements (data not shown).

Oligo(dT)s were also completely separated into seven bands in capillary gel electrophoresis without PVAd (Figure 1-4A). However the electrophoretic profiles of oligo(dT)s were drastically changed when PVAd was immobilized in gel matrix (Figure 1-4B, C). The migration times of oligo(dT)s increased as either the concentration of immobilized PVAd or the length of oligo(dT) increased. At 0.2 % PVAd, all of the oligo(dT)s did not migrate at all (Figure 1-4C). This is probably due to strong binding of oligo(dT) to PVAd immobilized gel.

Effect of the molecular weight of PVAd on the migration of oligo(dA)s and oligo(dT)s is summarized in Table 1. Under this condition, the migration of oligo(dA)s or (dT)$_{12}$ was unaffected by the presence of PVAd regardless the molecular weight of PVAd. However the migration times of (dT)$_{15}$ and (dT)$_{16}$ showed a significant increase

(A)

(B)

(C)

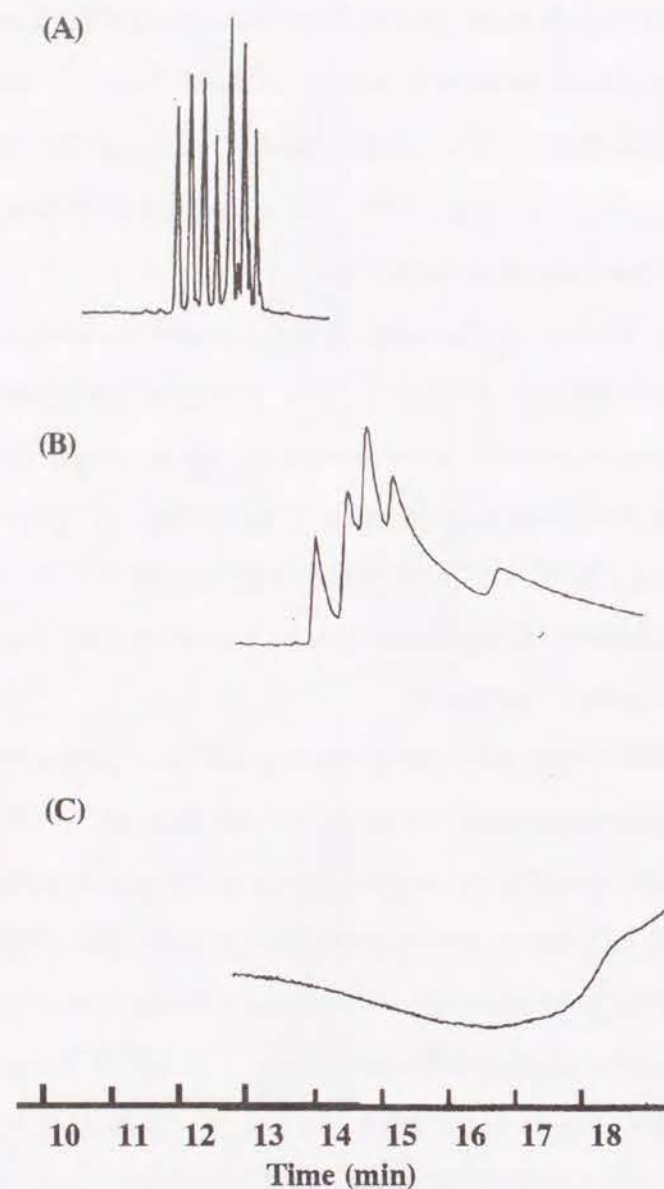10 11 12 13 14 15 16 17 18
Time (min)

Figure 1-4. Separation of (dT)$_{12-18}$ in capillaries filled with acrylamide gel or PVAd-immobilized gel. Concentration of immobilized PVAd: (A) 0 %, (B) 0.05 %, and (C) 0.2 %. Electrophoretic conditions are same as in Figure 1-3.

with PVAd of higher molecular weight. The retardation of the migration time was more significant for (dT)$_{16}$ than (dT)$_{15}$.

Table 1-1 Migration time of oligodeoxynucleotides in capillary affinity gel electrophoresis using PVAd with different molecular weights

Oligonucleotides	Migration time (min)			
	Without PVAd	PVAd with molecular weight of:		
		$< 10^4$	$1 \sim 3 \times 10^4$	$3 \sim 5 \times 10^4$
(dT)$_{12}$	13.20	13.50	14.26	13.76
(dT)$_{15}$	14.01	14.56	15.56	16.00
(dT)$_{16}$	14.35	14.84	17.62	20.87
(dA)$_{12}$	12.54	12.87	12.62	12.55
(dA)$_{15}$	13.34	13.72	13.40	13.31
(dA)$_{16}$	13.62	14.00	13.66	13.58

Concentration of PVAd was 0.05 %. Electrophoretic conditions are as in Figure 1-3.

The migration of oligo(dA)s became slower as the concentration of urea in the gel preparation and in the running buffer increased (Figure 1-5A). A similar dependence was also observed in the case without PVAd (data not shown). This increase of the migration time was consistent with the change of the viscosity of the buffer by increasing urea concentration (Figure 1-6). The change of migration time of (dT)$_{12}$ by the urea concentration was similar to that of oligo(dA)s (Figure 1-5B). In contrast the migration time of (dT)$_{15}$ and
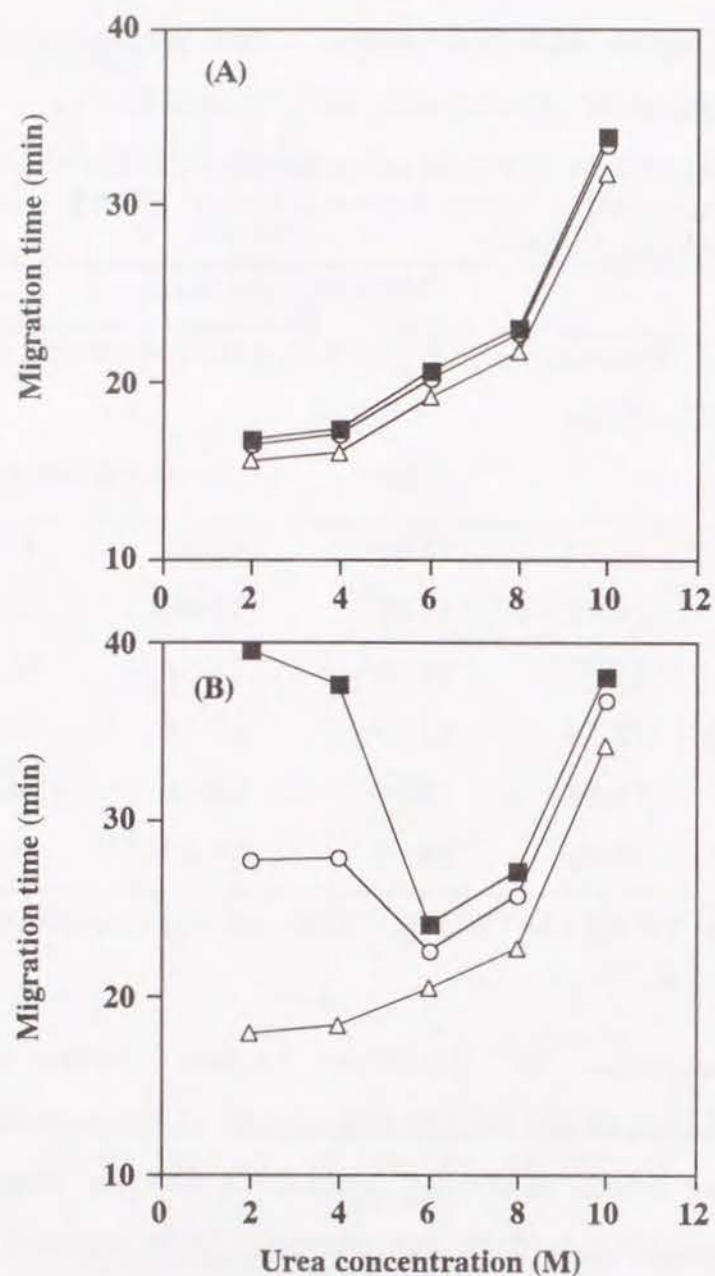
Figure 1-5. Effect of urea concentration in buffer on migration time of oligodeoxyadenylic acids (A) and oligothymidylic acids (B) in capillary affinity gel electrophoresis. Conditions are same as in Figure 1-3 except concentrations of urea and PVAd (0.02 %).
Chain length of deoxynucleotides : 12 (△), 15 (○), and 16(■).

$(dT)_{16}$ drastically decreased in the range of the urea concentration from 4.0 M to 6.0 M, then increased as the urea concentration became higher (Figure 1-5B).
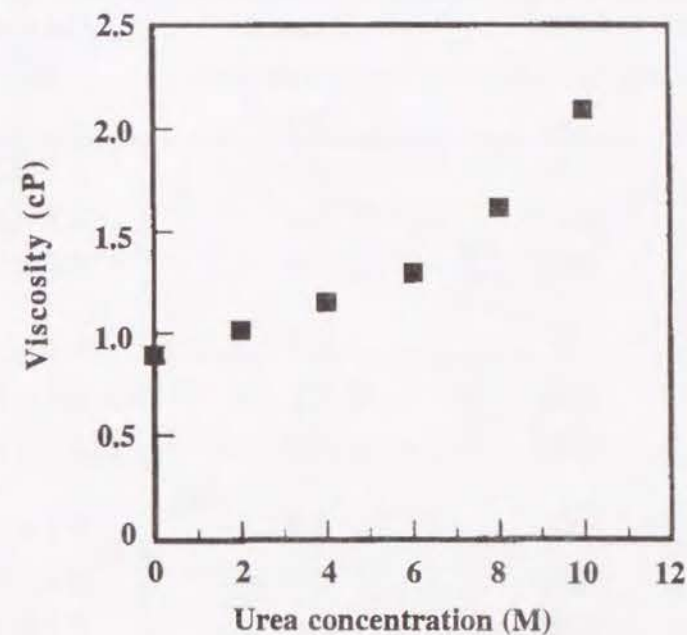

Figure 1-6. Viscosity change of 0.1 M Tris-borate buffer as a function of urea concentration at 25 °C.

The effect of PVAd and urea concentration on the separation of the oligonucleotides are summarized as the plate number in Table 2. For oligo(dT)s, the introduction of PVAd into gel matrix generally causes the decrease of the plate number. The decrease could be compensated by using higher concentration of urea. The plate number for $(dT)_{15}$ in the presence of PVAd drastically increased as the concentration of urea increased. The plate number for $(dT)_{15}$ with 6.0 M urea was 28 times larger than that with 4.0 M urea. In the case of oligo(dA)s, both the introduction of PVAd and the increase of the urea

concentration accompanied only a minor decrease of the plate number.

Table 1-2 Plate numbers for separation of oligodeoxy-
nucleotides by electrophoresis using capillaries filled with
PVAd immobilized gel

Oligonucleotides	PVAd concentration (%)	Urea concentration (M)	Plate number (m^{-1})
$(dT)_{12}$	0	4.0	5.7×10^5
	0.02	4.0	2.8×10^4
	0.02	6.0	4.6×10^5
$(dT)_{15}$	0	4.0	6.4×10^5
	0.02	4.0	1.2×10^3
	0.02	6.0	3.4×10^4
$(dA)_{12}$	0	4.0	5.1×10^5
	0.02	4.0	3.2×10^5
	0.02	6.0	1.2×10^5
$(dA)_{15}$	0	4.0	5.7×10^5
	0.02	4.0	5.5×10^5
	0.02	6.0	2.4×10^5

Electrophoretic conditions other than the concentration of PVAd and
urea are as in Figure 1-3.

Capillary temperature also affected the migration of
oligonucleotides. Figure 1-7 shows the relationship between the
migration time of oligonucleotides and capillary temperature with
0.05 % PVAd immobilized gel. The migration time of oligo(dA)
decreased as the capillary temperature increased. A similar effect of
the capillary temperature on the migration times of oligo(dA)s and
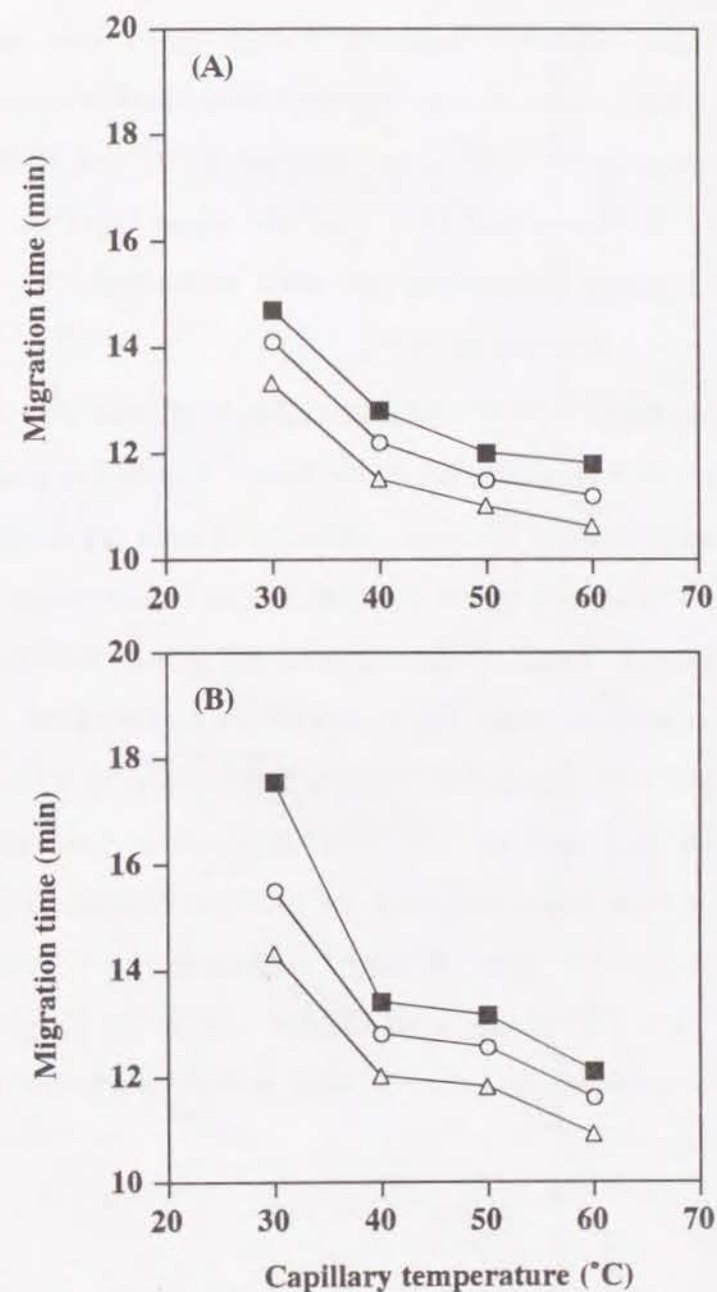
Figure 1-7. Effect of capillary temperature on migration time of
oligoadenylic acids (A) and oligothymidylic acids (B) in capillary affinity gel
electrophoresis with 0.05 % PVAd. Conditions are same as in Figure 1-3
except capillary temperature. Chain length of oligodeoxynucleotides : 12
($\triangle$),15 ($\bigcirc$), and 16($\blacksquare$).

oligo(dT)s was also observed without PVAd (data not shown). Although the migration times of oligo(dT)s also decreased with increase of the capillary temperature, the change between 30 °C and 40 °C was more drastic than that of oligo(dA)s. At the same time the plate number for the oligo(dT) separation was also increased. The plate number for $(dT)_{15}$ was 1×10^5 /m at 50 °C.

A capillary filled with PVAd immobilized gel was effective for base specific separation of oligodeoxynucleotides. Figure 1-8 compares the separation of a mixture of $(dA)_{12-18}$ and $(dT)_{15}$ using a capillary filled with conventional acrylamide gel to that filled with PVAd immobilized gel. In electrophoresis using a conventional gel filled capillary, the peak of $(dT)_{15}$ appeared between $(dA)_{17}$ and $(dA)_{18}$ and was not clearly resolved from those two oligo(dA)s (Figure 1-8A). Using a capillary filled with PVAd immobilized gel and controlling the capillary temperature, $(dT)_{15}$ was separated from the mixture of $(dA)_{12-18}$ (Figure 1-8B). The resolution was further improved by optimizing concentrations of both PVAd and urea (Figure 1-8C). In Figure 1-8C the separation was achieved less than 32 min, and the plate number for $(dT)_{15}$ was 5×10^5 /m.
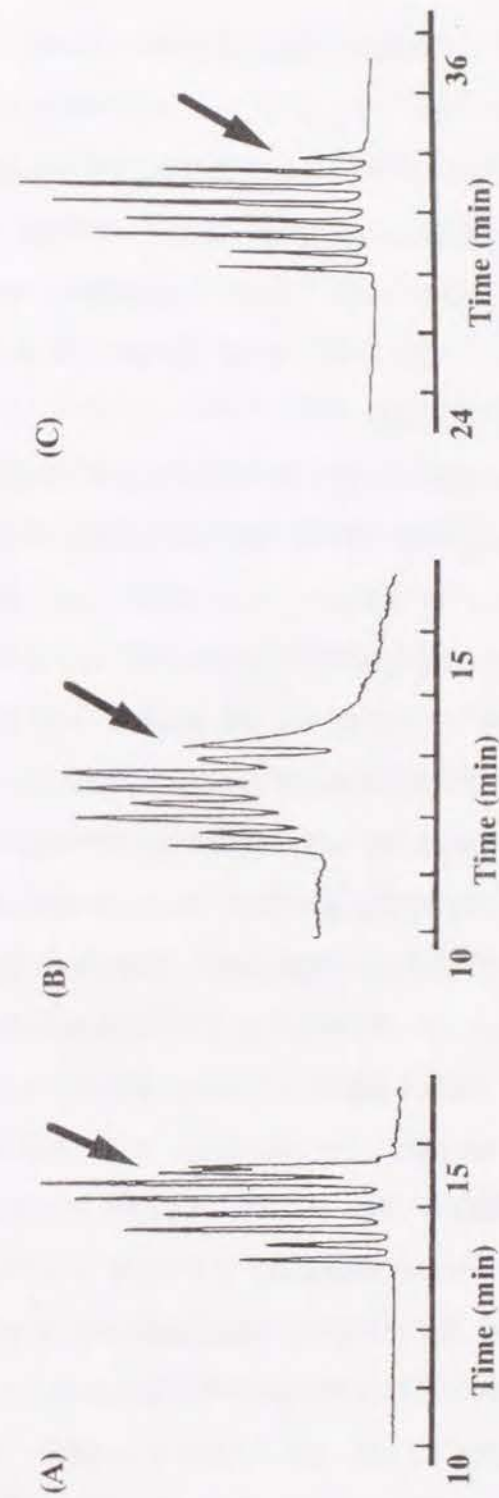
Figure 1-8. Capillary affinity gel electrophoresis of a mixture of $(dA)_{12-18}$ and $(dT)_{15}$. Arrow indicates the peak of $(dT)_{15}$. (A) Conventional capillary gel electrophoresis without PVAd in gel matrix. (B) Temperature programmed capillary affinity gel electrophoresis with 0.1 % PVAd. Capillary temperature: 50 °C from 0 to 4 min and 60 °C from 4 to 20 min. (C) Capillary affinity gel electrophoresis with 0.02 % PVAd and 8 M urea.

DISCUSSION

Capillaries filled with polyacrylamide gel immobilizing PVAd showed stability comparable to that without PVAd. The excellent reproducibility confirmed in the repetitive separation of oligodeoxynucleotides in the present study proves that CAGE with PVAd ligands is highly reliable.

PVAd immobilized in gel does not significantly interact with oligo(dA)s. The presence of PVAd did not affect the electrophoretic profile of oligo(dA)s including the peak shape and the mobility. In contrast, the migration of oligo(dT)s became slower by immobilization of higher concentration of PVAd in gel matrix and for longer chain length of oligo(dT)s. These results suggest that the retardation of oligo(dT) migration should be attributed to the specific interaction between PVAd and oligo(dT)s as also seen in the case of AGE of polynucleotides using PVAd immobilized slab gel supports.[3,4]

In analogy with the complementary hydrogen bonding known in a DNA duplex, the interaction between PVAd and oligo(dT)s is expected to be based on hydrogen bonding. The observed dependence of the migration behavior of oligo(dT)s to the urea concentration and the capillary temperature further supports this view.

The effect of the urea concentration and of the capillary temperature to the mobility of oligo(dA)s may be attributed to the change of the viscosity of the gel. Mobility of an electrophoresed substance is inversely proportional to the viscosity of the medium.[8]

In the case of oligo(dT)s, the migration times of oligo(dT)$_{15}$ and oligo(dT)$_{16}$ first decreased and then increased as the concentration of urea in running buffer increased. This behavior indicates that urea should have two possible effects on the migration of oligo(dT)s counteracting each other. One is to increase medium viscosity, that explains increase of the migration time above 6.0 M urea. The other one is to decrease the interaction between PVAd and oligo(dT)s, which becomes significant at urea concentration below 6.0 M.

The increase of capillary temperature causes a decrease of the viscosity of gel, resulting a increase of the mobility of oligodeoxynucleotides. The more drastic change of the migration time observed for oligo(dT)s than for oligo(dA)s between 30 °C and 40 °C indicates weakening of the interaction specific to oligo(dT)s by higher temperature. Increase of temperature or urea concentration is known to cause the destruction of hydrogen bonding.[9] These observations suggests strongly hydrogen bonding as a plausible interaction between PVAd and oligo(dT)s in CAGE separation.

Similar to the observation in the present study using CAGE, in the previous studies of the interaction between PVAd and poly(U) in aqueous bulk media, the thermodynamic stability of a PVAd-poly(U) complex became higher with increasing of the molecular weight of PVAd.[10] However the basis for this effect of the molecular weight of PVAd on the stability of PVAd-oligonucleotide complexes still remains unclear.

The migration times of oligo(dA)s which have little interaction

with PVAd in CAGE with PVAd ligands were almost identical to that without ligand. This result suggests that gel matrices of a similar pore size were generated in both of the capillaries regardless the presence of PVAd. This is one of the essential points in the preparation of a gel matrix which immobilizes an affinity ligand for AGE because, in evaluation of the interaction between an affinity ligand and an analyte, the effect of the interaction on the electrophoretic behavior of the analyte is to be singled out by comparing results obtained with and without the ligand.[1,2] (see Chapter 2) Contribution of factors other than the affinity interaction, such as sieving effect of gel, to difference between the two electrophoretic behaviors has to be negligible.

The present study demonstrated that a capillary for CAGE with immobilized affinity ligands can easily be prepared by a simple procedure. In principle, the preparation does not involve any process which is particular to the current PVAd system. Therefore the same protocol should be applicable to other combinations of ligands and analytes.

The results described above show that the separation in the CAGE system can be flexibly controlled by adjusting the concentrations of PVAd and urea, and capillary temperature. This allows fine optimization of the separation condition as demonstrated by the base line resolution of $(dT)_{15}$ from oligo(dA)s. Here, one can see that the excellent combination between high resolution of CGE and affinity separation of AGE is materialized in the present CAGE system. The

CAGE system is quite sensitive to a small difference in the interaction between PVAd ligands and oligodeoxynucleotides. This aspect of the system is further explored in the study of the effect of a mismatching base in an oligonucleotide sequence on the interaction with PVAd described in Chapter 2.

REFERENCES

[1]   Horejsi, V. and Ticha, M. (1981) *J. Chromatogr.*, 216, 43-62.

[2]   Horejsi, V. and Ticha, M. (1986) *J. Chromatogr.*, 376, 49-67.

[3]   Yashima, E., Suehiro, N., Miyauchi, N. and Akashi, M. (1993) *J. Chromatogr.*, 654, 151-158.

[4]   Yashima, E., Suehiro, N., Miyauchi, N. and Akashi, M. (1993) *J. Chromatogr.*, 654, 159-166.

[5]   Akashi, M., Iwasaki, H., Miyauchi, N., Sato, T., Sunamoto, J. and Takemoto, K. (1989) *J. Bioact. Compt. Polym.*, 4, 124-136.

[6]   Baba, y., Matsuura, T., Wakamoto, K., Morita, Y., Nishitsu, Y. and Tsuhako, M. (1992) *Anal. Chem*, 64, 1221-1225.

[7]   Foley, J. P. and Dorsey, J. G. (1983) *Anal. Chem*, 55, 730.

[8]   Kleparnik, K. and Bocek, P. (1991) *J. Chromatogr.*, 569, 3.

[9]   Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) In *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Plainview, 2nd ed.

[10]   Yashima, E., Tajima, T., Miyauchi, N. and Akashi, M. (1992) *Biopolymers*, 32, 811-817.

Chapter 2

Interaction between nucleic acids and its vinylanalogue, poly(9-vinyladenine)

In the previous chapter, a capillary affinity gel electrophoresis (CAGE) system was constructed for separation of oligonucleotides as well as evaluation of specific interaction between poly(9-vinyladenine) (PVAd) and oligonucleotides. The reliability and the basic characteristics of the CAGE system were demonstrated with simple homooligonucleotides. In this chapter, the CAGE system was applied to a more complicated example; oligonucleotides bearing heterogeneous nucleobases.

In a native DNA duplex, mismatching among bases are known to destabilize the duplex.[1] In order to study a similar effect of mismatching on the interaction between PVAd and oligonucleotides, hexadeoxynucleotides bearing thymidylic acid-deoxyadenylic acid sequences (dT-dA hexamers) were systematically synthesized as the model sequence. Using the CAGE system, apparent association constant was obtained for the complexation between PVAd and the dT-dA hexamers.

Moreover, the present CAGE system was applied also for separation of native DNA. Specific recognition of a single base

mutation on partial sequences of *ras* oncogene was investigated by this CAGE system.

MATERIALS AND METHODS

Materials

Cytosine 5'-monophosphate (CMP) was purchased from Sigma (St. Louis, MO, U.S.A). All oligodeoxynucleotides used in this study were synthesized using a Model 391 DNA synthesizer (ABI, Foster City, CA). PVAd used in this experiment has its molecular weight in the range from 10,000 to 30,000. All other reagents and materials including buffers were the same as those used in Chapter 1. The preparation of gel-filled capillaries was also described in Chapter 1.

Capillary gel electrophoresis

Capillary electrophoresis was run on a CE-800 capillary electrophoretic system (Jasco, Tokyo) with a buffer solution containing 4.0 M urea at 9 kV (214 V/m). Samples those contain CMP as the internal standard were electrophoretically injected into the capillary by applying 5 kV for 0.1 s. All the oligodeoxynucleotides were detected at 260 nm. Electropherograms were processed on a CR-3A integrator (Shimadzu, Kyoto). Capillary temperature was kept at 20 ± 0.5 °C.

Theory

The apparent association constant (Ka) for complex formation between a nucleic acid (N) and PVAd (L) is expressed as equation 1.

$$N + L \leftrightarrows N \cdot L \qquad Ka = [N \cdot L] / [N] [L] \qquad (1)$$

where $N \cdot L$ indicates the complex, and the brackets represent the concentration of respective species. In the present study, Ka was obtained in accordance with the established theory of affinity electrophoresis.[3]

For electrically neutral PVAd having appropriate size (considered as 20,000 in this study), the mobility of the PVAd-nucleic acid complex could be regarded as zero. In this case, the relationship between the mobility and the concentration of nucleic acid can be given by equations 2 and 3.

$$\frac{\mu_0}{\mu} = \frac{[N]_{total}}{[N]} \qquad (2)$$

$$[N]_{total} = [N] + [N \bullet L] \qquad (3)$$

where μ_0 and μ are respective mobilities of a given nucleic acid in the absence and presence of PVAd. Combining equations 1, 2 and 3 yields equation 4.

$$\frac{\mu_0}{\mu} = 1 + Ka[L] \qquad (4)$$

The concentration of free PVAd ([L]) is practically equal to the total concentration of PVAd in the gel ([L]$_{total}$) when [L]$_{total}$ is much higher than the total concentration of nucleic acid. The migration time t is

inversely proportional to the mobility. Equation 4 is expressed as equation 5.

$$t = t_0(1 + Ka[L]_{total}) \qquad (5)$$

where t_0 is the migration time of the nucleic acid without PVAd ($[L]_{total}$ = 0). In this study, the migration time of nucleic acid at various concentrations of PVAd is experimentally determined, and Ka is obtained from the slope of the t/t_0 vs. $[L]_{total}$ plot.

RESULTS

Interaction of PVAd with homodeoxynucleotide hexamers

In Chapter 1, the interaction between PVAd and two homodeoxyoligonucleotides, deoxyadenylic acid (dA) and thymidylic acid (dT), was studied using a capillary affinity gel electrophoresis (CAGE) with PVAd immobilized. Investigation was extended to the cases with deoxyguanylic acid (dG) and deoxycytidylic acid (dC). A typical electropherogram of a mixture of four different homodeoxy-nucleotide hexamers with or without PVAd immobilized is shown in Figure 2-1. Only for $(dT)_6$, the relative migration time to the internal standard (CMP) became longer in the presence of PVAd. Peaks with the other three hexamers appeared at almost the same positions as that observed with CMP regardless of the presence of PVAd.
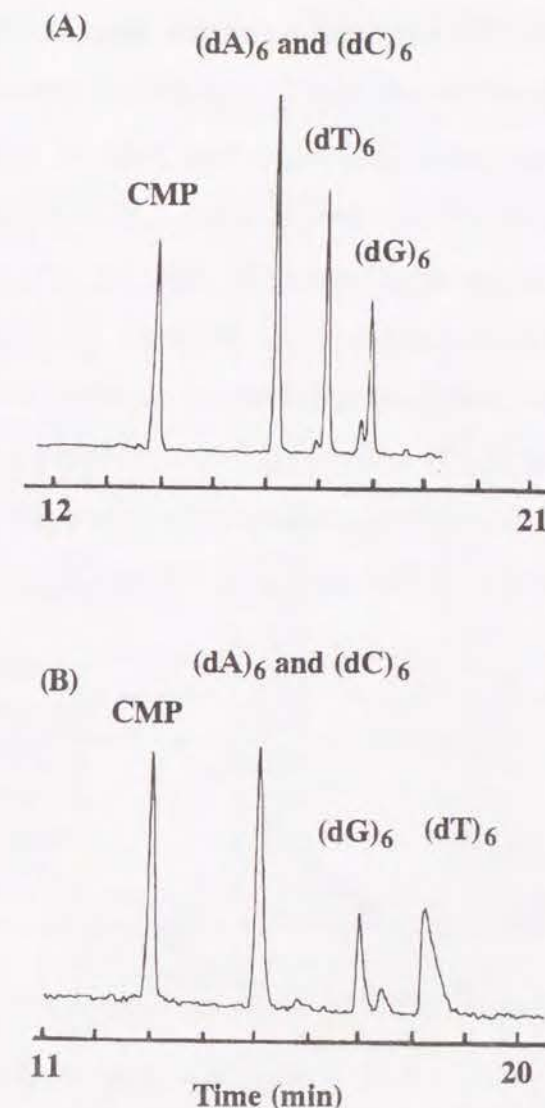
Figure 2-1. Electrophoresis of homodeoxynucleotide hexamers. (A) CGE without PVAd. (B) CAGE with 0.2 % PVAd. Running buffer: 0.1 M Tris-borate and 4.0 M urea (pH=8.6). Capillary temperature: 30 °C. Other conditions are described in Materials and Methods.

The relative migration time of the hexadeoxynucleotides with various concentrations of PVAd was investigated, and the result is

shown in Figure 2-2. The relative migration time of $(dA)_6$, $(dC)_6$, and $(dG)_6$ was not significantly affected by the PVAd concentration up to 0.4 %. On the other hand the migration time of $(dT)_6$ drastically increased linearly to the PVAd concentration. This linear relationship is consistent with the assumption for the complex formation according to equation 1 (correlation coefficient of 0.95 in the least-square method). Using equation 5, the apparent association constant of complexation, Ka, was calculated as listed in Table 2-1 (1-4). The Ka of PVAd-$(dT)_6$ complex was approximately 7 times larger than that of PVAd-$(dG)_6$ and 80 times larger than that of PVAd-$(dA)_6$ or PVAd-$(dC)_6$.
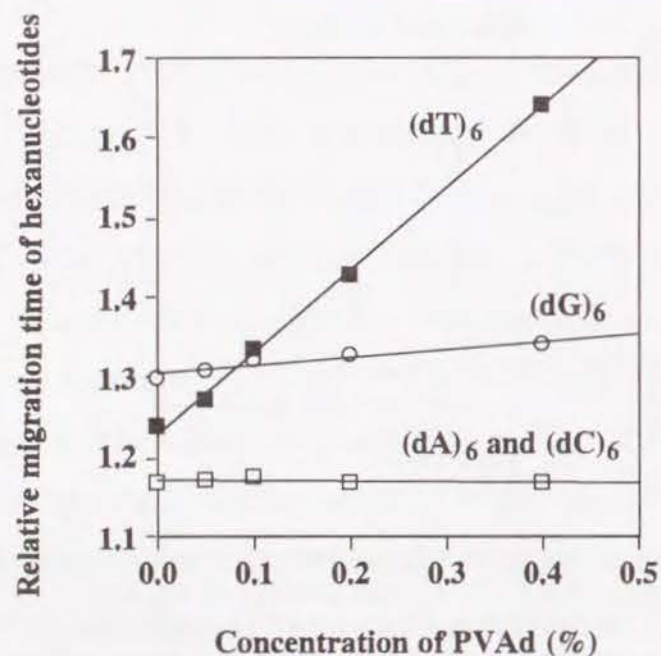
Figure 2-2. Migration time of homodeoxynucleotide hexamers relative to CMP in CAGE separation as a function of the concentration of PVAd in gel. $(dA)_6$ and $(dC)_6$ remained unresolved under this condition. Electrophoretic conditions except PVAd concentration are same as in Figure 2-1.

Table 2-1. Apparent Ka for PVAd-oligodeoxynucleotide complexation

| | Base sequence (5' to 3') | Relative migration time to CMP | | Ka (mol^{-1}dm^3) |
		without PVAd (t_0)	with PVAd (t)	
(1)	AAAAAA	1.170	1.176	500 ± 300
(2)	GGGGGG	1.297	1.357	5900
(3)	CCCCCC	1.170	1.176	500 ± 300
(4)	TTTTTT	1.239	1.627	39800 ± 800
(5)	TAAAAA	1.177	1.185	800 ± 300
(6)	TTAAAA	1.193	1.203	1100 ± 300
(7)	TTTAAA	1.202	1.233	3200 ± 400
(8)	TTTTAA	1.210	1.280	7100 ± 700
(9)	TTTTTA	1.228	1.442	22300 ± 1200
(10)	TTTTAT	1.228	1.364	13900 ± 930
(11)	TTTATT	1.228	1.336	10800 ± 970
(12)	TTTATA	1.210	1.690	6580
(13)	TTATTA	1.210	1.662	6330
(14)	TTTAAT	1.210	1.500	4000
(15)	TTATAT	1.210	1.500	4000

All the runs except for (10) - (15) were triplicated. Concentration of PVAd was 0.4% (row (1) - (9)), 3.0% (row (10) - (15)). Electrophoretic conditions are same as in Figure 2-1.

Interaction between PVAd and hexadeoxynucleotides containing different lengths of thymidylic acid units

Hexadeoxynucleotides those had thymidylic acid and adenylic acid (dT-dA hexamers) at various ratios were synthesized. Typical electrophoretic profile of a mixture of dT-dA hexamers those have a T sequence from zero to six is shown in Figure 2-3. In the presence of PVAd, significant retardation in the migration was observed with the dT-dA hexamers of which the T sequence was longer than 3.
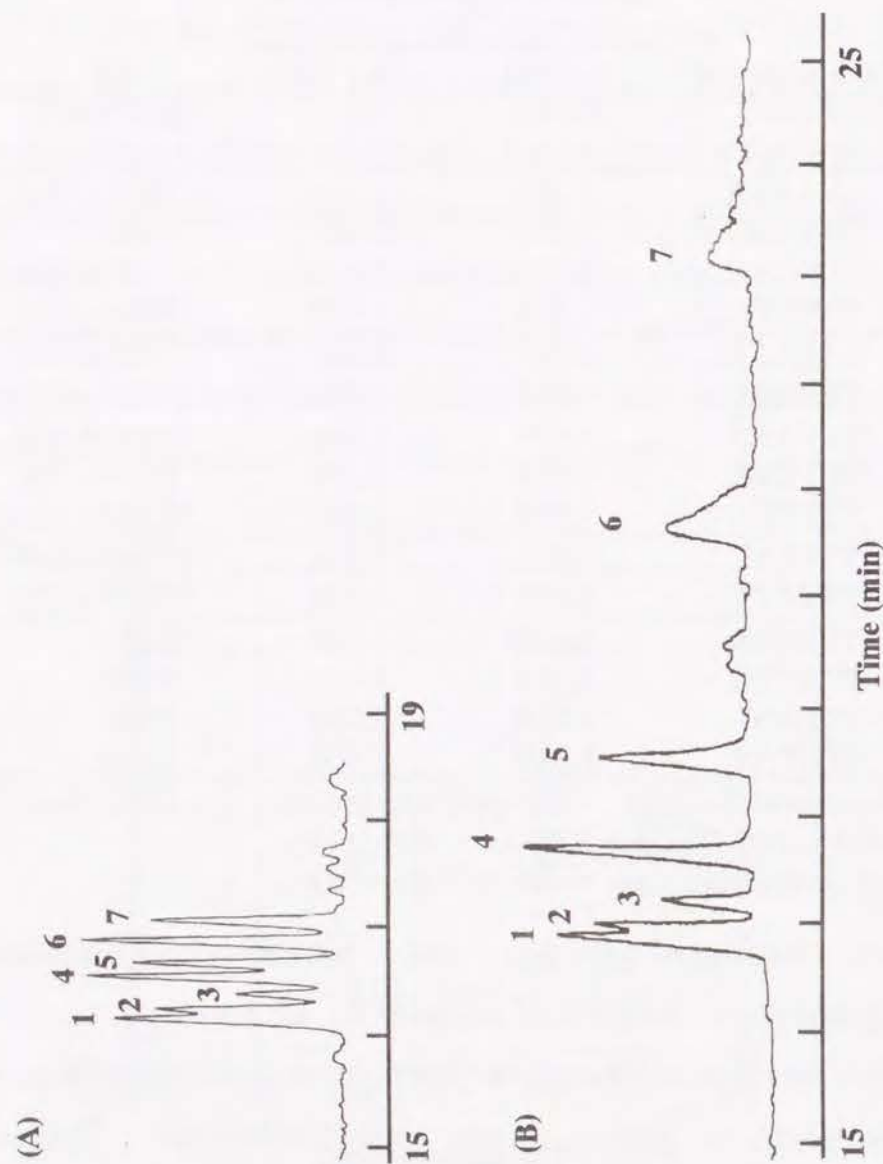
Figure 2-3. Separation of a mixture of dT-dA hexamers containing a T sequence of different length in CGE (A) and in CAGE with 0.4 % PVAd (B). Nucleotides: 1;AAAAAA, 2;TAAAAA, 3;TTAAAA, 4;TTTAAA, 5;TTTTAA, 6;TTTTTA, 7;TTTTTT. Electrophoretic conditions except PVAd concentration are same as in Figure 2-1.
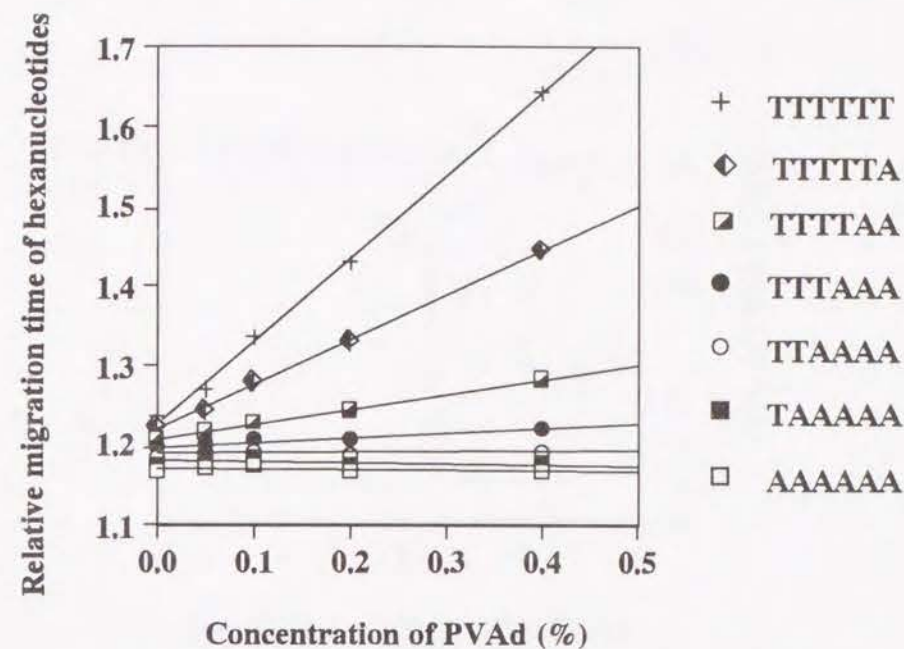

Figure 2-4. Migration times of dT-dA hexamers relative to CMP in CAGE as a function of the concentration of PVAd. Electrophoretic conditions except PVAd concentration are same as in Figure 2-1.

The relative migration time of the dT-dA hexamers was measured at different PVAd concentrations as shown in Figure 2-4. The Ka-values for the complexation of the hexamers with PVAd are summarized in Table 2-1 (5-9). The change of the standard free energy ($\Delta G°$) for the complex formation is calculated using equation 6.

$$\Delta G = -RT \ln Ka \qquad (6)$$

where R is the gas constant and T is the temperature (293 K in the present study). The results are related with the length of T sequence (Figure 2-5). The $\Delta G°$ decreased as the length of T sequence increased.
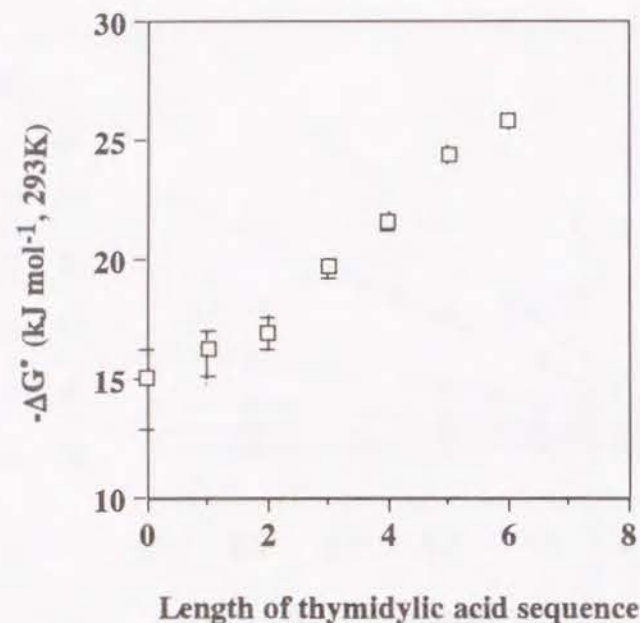
Figure 2-5. Free energy change for complex formation between PVAd and deoxynucleotide hexamers as a function of the length of thymidylic acid sequence.

Interaction between PVAd and hexadeoxynucleotides having various thymidylic acid sequences

T5A1 dT-dA hexamers were designed by systematically replacing one thymidylic acid unit by an adenylic acid unit in $(dT)_6$ sequence as an example of base mismatch. There are six different T5A1 hexamers, namely, 5'-TTTTTA-3', TTTTAT, TTTATT, TTATTT, TATTTT, and ATTTTT.

Figure 2-6 shows the electrophoretic profile of a mixture of $(dT)_6$ and three T5A1 hexamers, TTTTTA, TTTTAT, and TTTATT. In the absence of PVAd, the three T5A1s could not be resolved clearly, while $(dT)_6$ migrated separately from the T5A1s (Figure 2-6A).
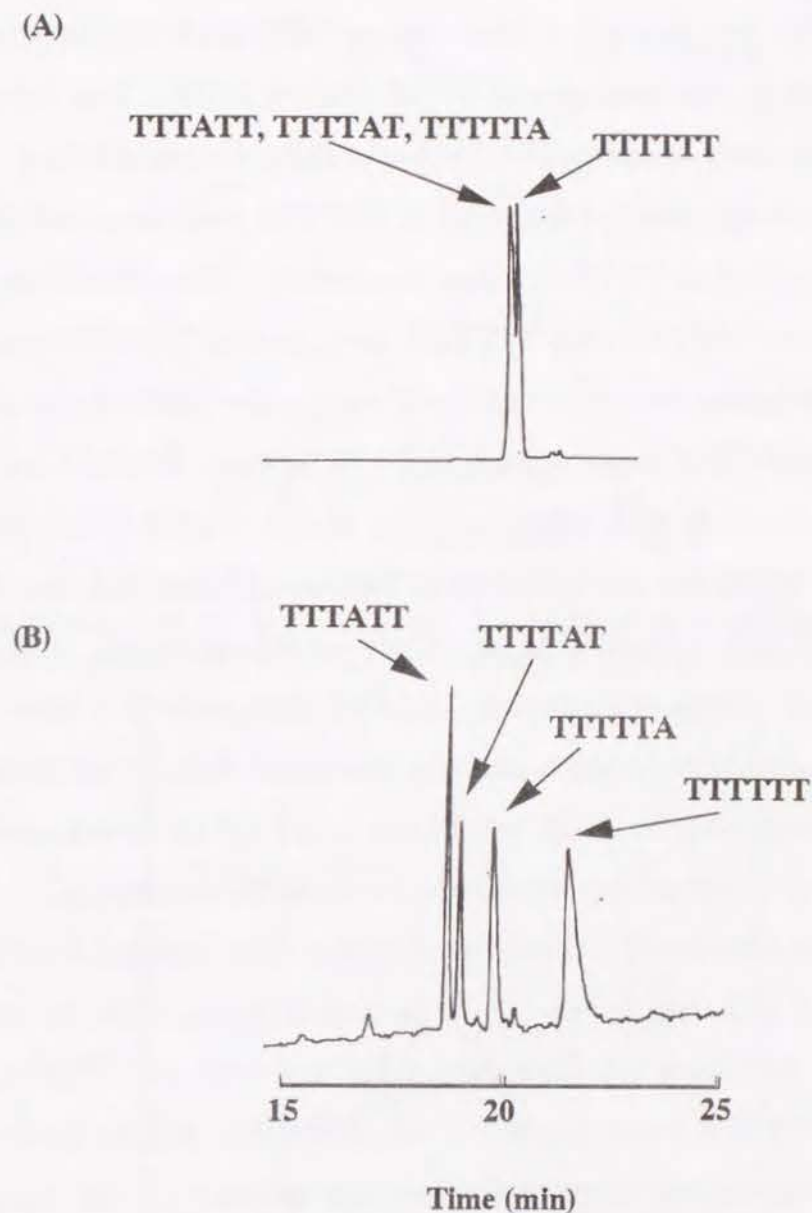
(A)

(B)

Figure 2-6. Electrophoresis of a mixture of $(dT)_6$ and T5A1 hexamers in CGE (A) and CAGE with 0.4 % PVAd.
Sample: 1; TTTATT, 2; TTTTAT, 3; TTTTTA, 4; TTTTTT.
Electrophoretic conditions are as in Figure 2-1.

The difference in the migration time among $(dT)_6$ and T5A1s was largely enhanced by the presence of PVAd (Figure 2-6B). The three T5A1s were completely separated each other in the presence of PVAd.

ATTTTT, the antiparallel sequence to TTTTTA, was migrated at the position identical to TTTTTA (data not shown). This is also the case for a pair of TATTTT and TTTTAT, or a pair of TTATTT and TTTATT. By reducing the concentration of urea in the buffer to 2.0 M and increasing the PVAd concentration to 2.0 %, however, TTTATT and TTATTT were resolved each other into two peaks (Figure 2-7). By optimizing the condition, the interaction between PVAd and the T bases increased, and it seemed to reveal the small difference in the antiparallel pair. However, although TTATTT that showed a longer migration time should have more strongly interacted with PVAd than TTTATT, the exact Ka's for these two T5A1s could not be determined due to the difficulty in complete separation between the two T5A1s.

The Ka's for the three T5A1s were calculated from Figure 2-8, and are summarized in Table 2-1 (9-11). The correlation coefficients for the fitted lines in Figure 2-8 were 0.97, 0.99, and 0.98 for TTTTTA, TTTTAT, and TTTATT, respectively. It was found that the Ka for the T5A1-PVAd complexation decreased when the position of the base mismatching shifted toward the center of the T5A1 sequence.
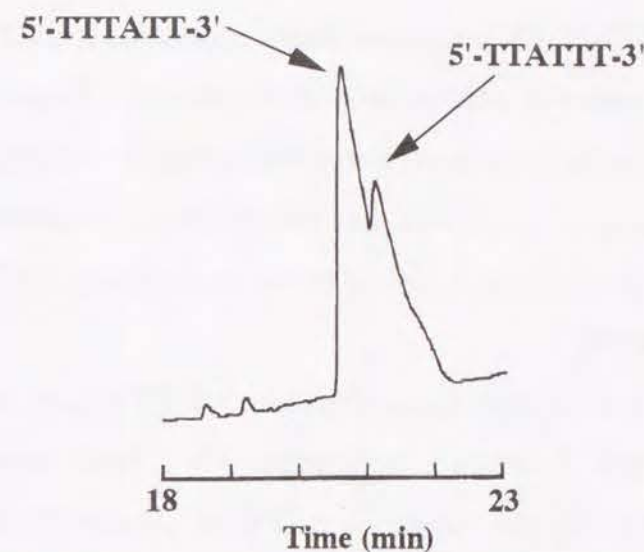

Figure 2-7. Separation of 5'-TTTATT-3' and 5'-TTATTT-3' in CAGE with 2.0 % PVAd in gel. Electrophoretic conditions except the concentrations of PVAd (2.0 %) and urea (2.0 M) are same as in Figure 2-1.
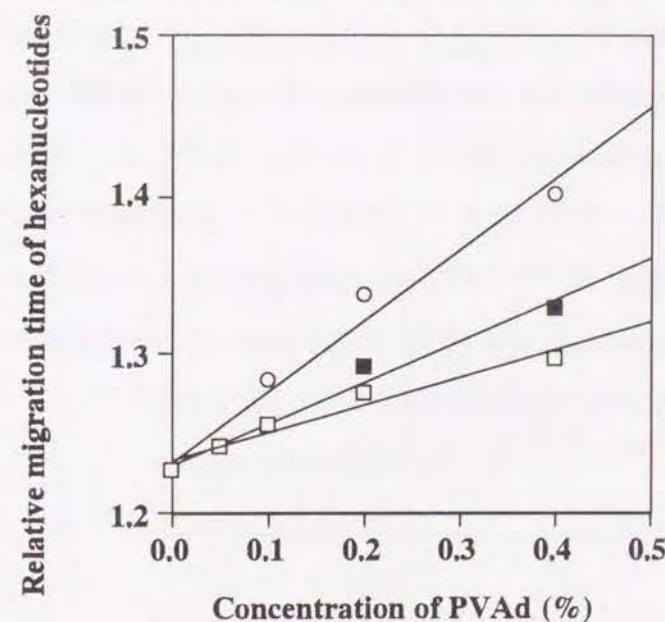

Figure 2-8. Migration times of dT-dA hexamers relative to CMP in CAGE as a function of PVAd concentraiton in gel. Nucleotides: TTTATT (□), TTTTAT (■), and TTTTTA (○) Electrophoretic conditions except the concentration of PVAd are same as in Figure 2-1.

Similarly, T4A2 dT-dA hexamers those consist of four thymidylic acid units and two adenylic acid units were examined. Three different T4A2s were designed to have a series of sequences in which one base mismatching occurred at the 3'-end and the other ones systematically shifted its position toward the center of the sequence, namely, TTTTAA, TTTATA, and TTATTA.

With a mixture of the three T4A2s and TTTAAA, a typical electrophoretic profile is shown in Figure 2-9. The three T4A2s completely separated in the presence of PVAd (Figure 2-9B). Two other T4A2s, in which one A was fixed at the position next to the 3'-end (TTTAAT and TTATAT), were also examined. However no resolution was seen between the two T4A2s under the condition employed. From Figure 2-10, the *Ka*'s for the complexation between PVAd and all five T4A2s were calculated as shown in Table 2-1 (8, 12-15). The order of *Ka*'s was TTTTAA > TTTATA ≈ TTATTA > TTTAAT ≈ TTATAT. Similarly to the case of PVAd-T5A1 complexation, with the case of PVAd-T4A2 complexation, the shift of the mismatching bases toward the center of a T4A2 sequence decreased the *Ka*-values.
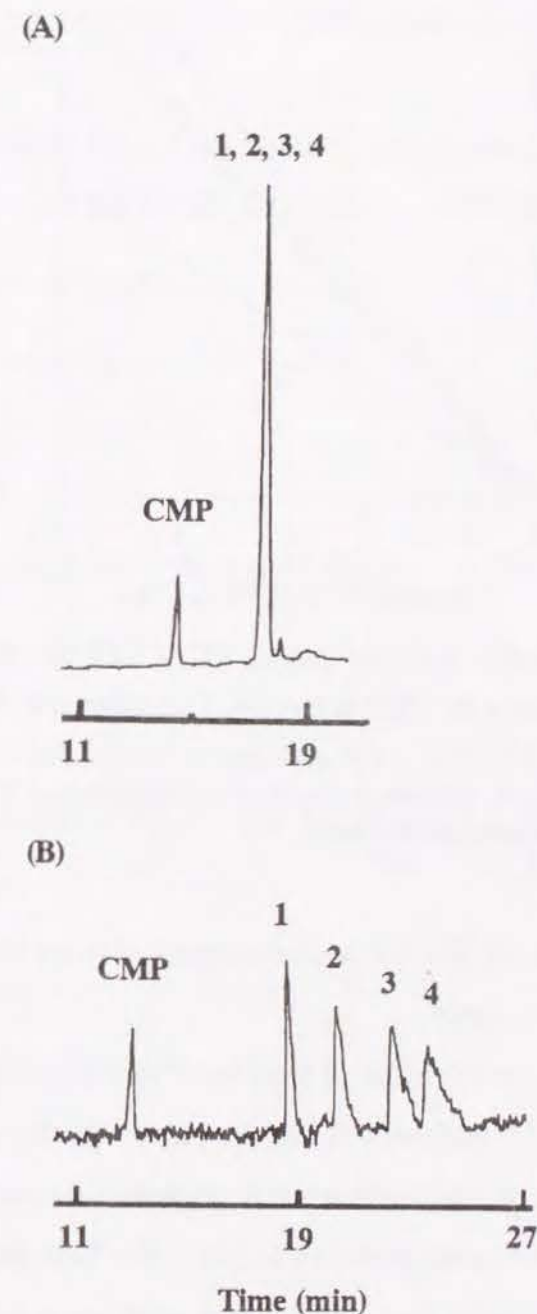
(A)

(B)

Time (min)

Figure 2-9. Separation of a mixture of TTTAAA and T4A2 hexamers in CGE (A) and CAGE with 3.0 % PVAd (B). Nucleotides: 1, TTTAAA; 2, TTATAT; 3, TTATTA; 4, TTTTAA. Electrophoretic conditions except PVAd concentration are same as in Figure 2-1.
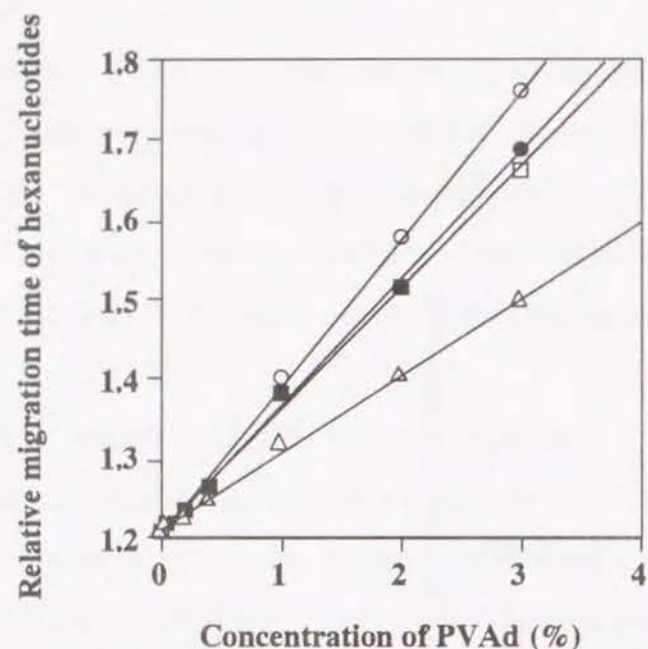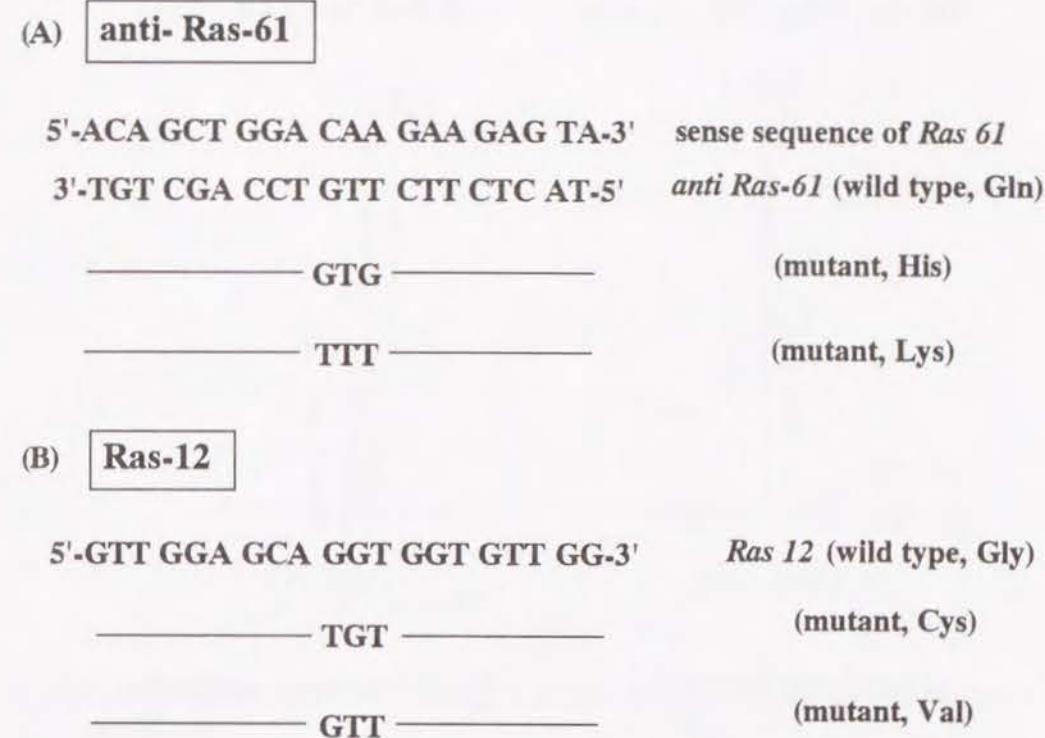
Figure 2-10. Migration times of T4A2s, TTTTAA(◯), TTTATA (●), TTATTA (☐) relative to CMP in CAGE. The migration times of TTTAAT and TTATAT (△) are also shown though these two T4A2s could not be resolved. Electrophoretic conditions except PVAd concentration are same as in Figure 2-1.

Interaction between PVAd and deoxynucleotides with native twenty-base sequences

The electrophoretic profile of a mixture of 20-mer DNAs, *anti-Ras 61* and its mutants, (Scheme 2-1A) is shown in Figure 2-11. Also given in Figure 2-12 is electrophoretic separation among other 20-mers, *Ras 12* and their mutants (Scheme 2-1B). For both cases of *anti-Ras 61* and *Ras 12*, the wild type and its mutants were migrated without any clear resolution in the absence of PVAd (Figure 2-11A and 12A). In contrast, the wild type and its mutants were completely separated into three bands by adding PVAd (Figure 2-11B and 12B).

(A) anti- Ras-61

5'-ACA GCT GGA CAA GAA GAG TA-3'   sense sequence of *Ras 61*
3'-TGT CGA CCT GTT CTT CTC AT-5'   *anti Ras-61* (wild type, Gln)

———————— GTG ————————   (mutant, His)

———————— TTT ————————   (mutant, Lys)

(B) Ras-12

5'-GTT GGA GCA GGT GGT GTT GG-3'   *Ras 12* (wild type, Gly)

———————— TGT ————————   (mutant, Cys)

———————— GTT ————————   (mutant, Val)

Scheme 2-1. Sequences of *Ras 61*, *anti Ras 61*, and *Ras 12* with their mutants.
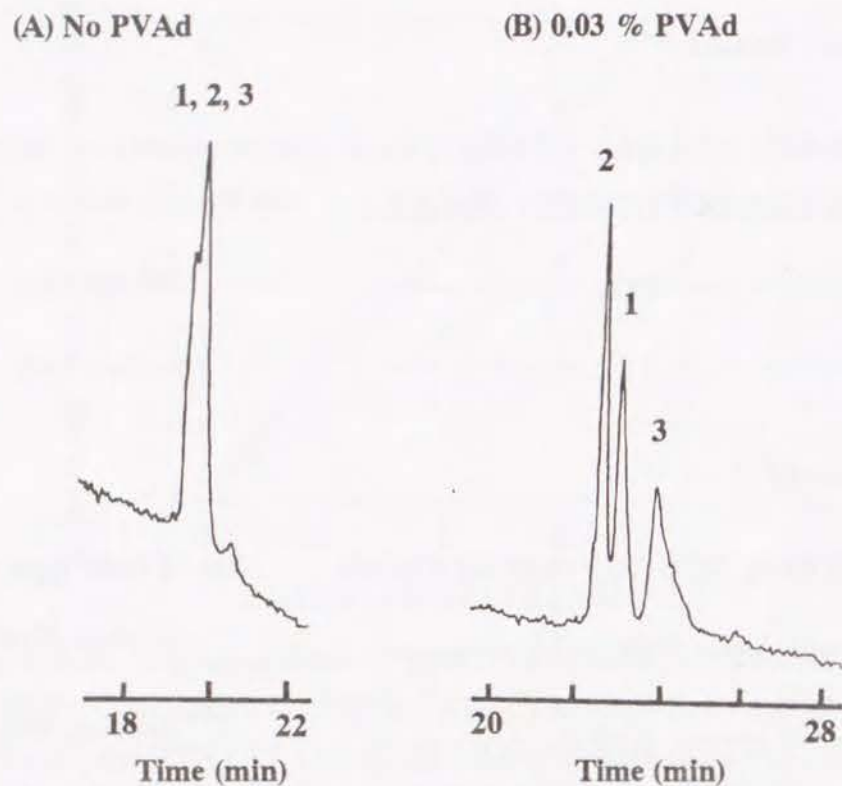
(A) No PVAd   (B) 0.03 % PVAd

Figure 2-11. Electrophoretic separation of a mixture of *anti-Ras 61* and its mutants.
DNA sequence:
1; 5'-TACTCTTC-TTG-TCCAGCTGT-3' (wild type, Gln),
2; 5'-TACTCTTC-GTG-TCCAGCTGT-3' (mutant, His), and
3; 5'-TACTCTTC-TTG-TCCAGCTGT-3' (mutant, Lys).
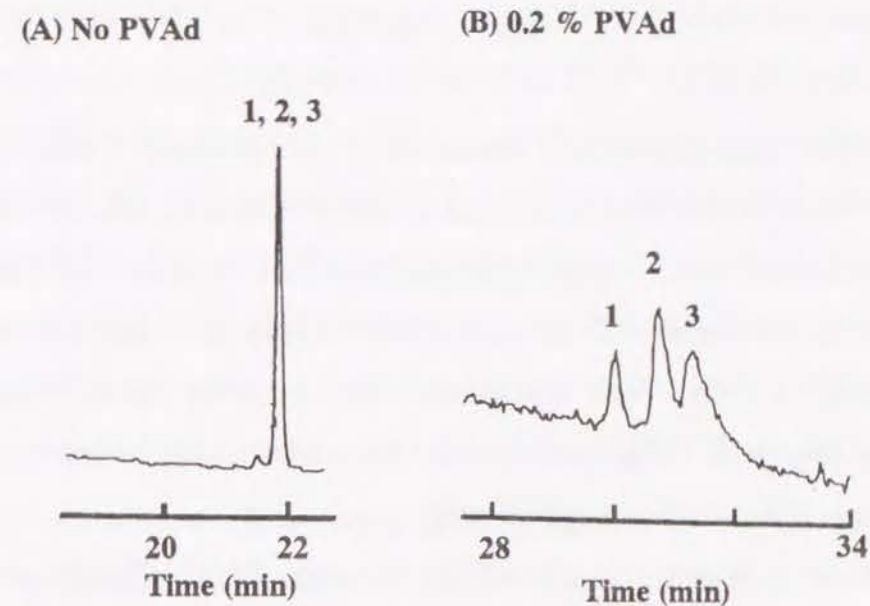Electrophoretic conditions except PVAd concentration are same as in Figure. 2-1.

(A) No PVAd   (B) 0.2 % PVAd

Figure 2-12. Electrophoretic separation of a mixture of *Ras 12* and its mutants.
DNA sequence:
1; 5'-GTTGGAGCA-GGT-GGTGTTGG- 3' (wild type, Gly),
2; 5'-GTTGGAGCA-TGT-GGTGTTGG- 3' (mutant, Cys), and
3; 5'-GTTGGAGCA-GTT-GGTGTTGG- 3' (mutant, Val).
Electrophoretic conditions except PVAd concentration are same as in Figure 2-1.

DISCUSSION

When homonucleotide hexamers of four different bases, G, T, A, and C were used, PVAd was found to specifically interact only with T to a significant extent. This result suggests that the major interaction in the complexation of a DNA with PVAd should occur between adenine moieties of PVAd and thymidylic acid units of DNA. The other nucleotides in a DNA sequence act as mismatching bases to weaken the interaction in the complexation.

In this study, the interaction between PVAd and those dT-dA hexamers examined showed a sequence specificity. The interaction seemed to obey two rules; (i) introduction of a mismatching base in place of T in the sequence of a hexamer decreases the interaction of the hexamer with PVAd, and (ii) a shift of a mismatching in a continuous T sequence toward the center of the T sequence further decreases the interaction.

A rather linear increase in the $-\Delta G°$ for the complexation with the longer T sequence (Figure 2-5) indicates that the contribution of the interaction between PVAd and T bases in the complexation is essentially additive. This is consistent with the first rule.

The dependence of the interaction between PVAd and a dT-dA hexamer to the sequence of the hexamer (the second rule) was similar to the dependence of the interaction between the two strands to their base sequences observed for intact DNA duplexes. For a DNA duplex, the introduction of mismatching base pairs into the sequence decreases

a thermodynamic stability of the duplex.[4-7] This destabilization has been studied by many researchers because of its close relevance to formation of a point mutation in DNA. At present the destabilization is mainly explained by a decrease in the nearest-neighboring stacking interaction (NNI, Scheme 2) by formation of a mismatching base pair.[4-7,8] Aboul-ela et al. reported that an A·A mismatching surrounded by (dT)n · (dA)n sequence destabilized a double helix DNA by approximately 1.5 kcal mol^{-1} (6.7 kJ mol^{-1}) in ΔG.[5] Werntges et al. proposed an additional long range effect of the mismatching, the next nearest neighboring interaction (NNNI).[6] This effect was, however, much smaller than that of NNI, estimated to be about 3 % of NNI.

A, B: complementary base pair

A, X: mismatching base pair
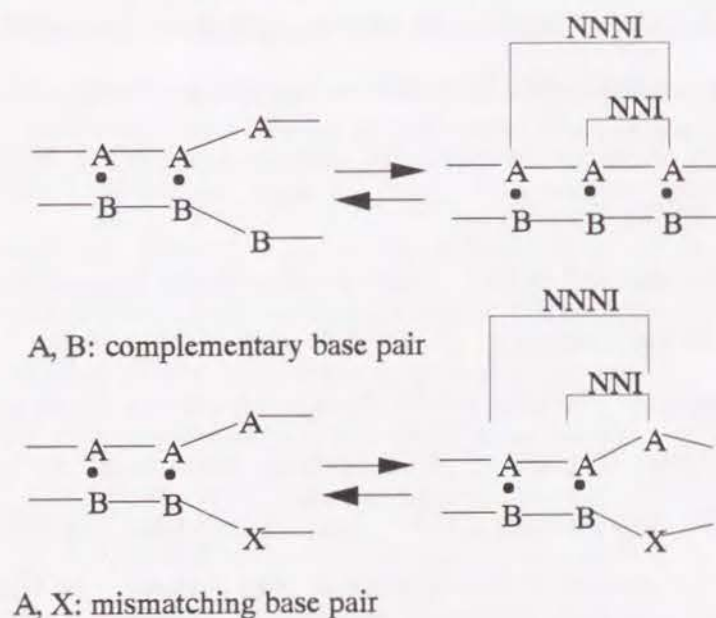
NNI: nearest neighbor interaction
NNNI: next nearest neighbor interaction

Scheme 2-1. Nearest neighbor interaction (NNI) and next nearest neighbor interaction (NNNI) in duplex formation.

The stronger interaction of PVAd with TTTTAT than that with TTTATT may be explained by considering NNNI in the complexation of PVAd and a dT-dA hexamer. There is no difference in the number of T units between the two sequences, TTTTAT and TTTATT. The two sequences have even the same number of the nearest neighboring stacking interactions (NNI). However, the difference is expected at the level of NNNI between the two sequences. TTTATT has one T(T)T, two T(T)A and one T(A)T NNNIs. On the other hand, TTTTAT, there are two T(T)T, one T(T)A and one T(A)T NNNIs. The nominal difference between the two hexamers in the free energy change for the complexation can be obtained by subtracting $\Delta G°$ of TTTTAT from that of TTTATT, 0.6 kJ mol^{-1} at 293K. In this explanation, this difference ($\Delta\Delta G°$) should correspond to the destabilization of the complex between PVAd and a dT-dA hexamer by replacing the stronger T(T)T NNNI by the weaker T(T)A NNNI.

The two rules observed in this study for the dT-dA hexamers were applicable also to a complex of PVAd and DNA that has a heterogeneous sequence. In this study, deoxynucleotides those consist of 20 bases and code a part of *Ras* protein were used as model sequences of naturally occurring DNA. *Ras 61* contains the codon for the position 61 in the amino acid sequence of *Ras* protein. In the wild type of *Ras*, the codon is CAA (glutamine). The mutation of this codon to either CAC (histidine) or AAA (lysine) is frequently observed. Anti-*Ras 61* is a single strand antisense DNA to *Ras 61*. With the mutation, the sequence of the antisense strand also changes from GTT

to either GTG or TTT. This relationship is shown in Scheme 2-1.

Table 2-2 Free energy change for PVAd-hexadeoxy-nucleotide complexation

hexadeoxynucleotide	$-\Delta G°$ (kJ mol^{-1}) at 293 K
TTTTTT	25.8
TTTTTA	24.4
TTTTAT	23.2
TTTATT	22.6
TTTTAA	21.6
TTTATA	21.4
TTATTA	21.3

$-\Delta G°$ was calculated from Ka listed in Table 2-1.

A mutation also occurs at the codon that codes the amino acid 12 (glycine) in the wild type of *Ras*. The sequences of *Ras 12*, which contains the 12th codon, is also described in Scheme 2-1. The mutation of *Ras 12* changes the codon GGT to either TGT or GTT.

When six-base sequences are chosen around the mutation point so that the sequences contain the maximum number of T bases, the order of the migration time can be expressed as GTGCTT < GTTCTT < TTTCTT for *anti-Ras 61*. This obeys the first rule that a sequence having less mismatching bases, G and C in this case, shows stronger interaction with PVAd. In the present case for *anti-Ras 61*, another choice of the six base sequence is possible. The alternative gives GTGCTT < GTTCTT < TTTCTT, which also agrees with the first rule.

In the case of *Ras 12*, a similar consideration yields GGTGGT < TGTGGT < GTTGGT. The weaker interaction of GGTGGT than the two other sequences also follows the first rule. The stronger interaction of PVAd with GTTGGT than that with TGTGGT obeys the second rule. The base composition of *anti-Ras 61*, $(dA)_2(dT)_9(dG)_3(dC)_6$, is totally different from that of *Ras 12*, $(dA)_2(dT)_6(dG)_{11}(dC)_1$. In addition, in these two sequences, guanylic acid units acted as the mismatching bases instead of adenylic acid units. The result so obtained suggests that the rules would be common to various complexes of PVAd and DNA oligomers regardless of their base composition or the kind of mismatching bases.

Except native nucleic acids, the destabilization of a complex with DNA accompanied by a mismatching base pair has been known only for a peptide type nucleic acid analog. The present CAGE study revealed that a similar effect of mismatching bases can also appear in complexation between nucleic acids and much simpler synthetic polymers. It should be noted that, even with PVAd, the effect of mismtching bases is not observed in any system other than CAGE. It is suprising that, with CAGE, such a simple synthetic polymer that has a plain poly(vinyl) backbone and homogeneous unit composition can distinguish a small difference in the base sequence of oligonucleotide.

The present study demonstrates the validity of CAGE as a method for the separation of oligonucleotides. The CAGE system examined here was sensitive enough to distinguish 20-mer oligonucleotides different only by one base pair. Point mutations of a single base pair

in gene are often associated with both inherited and acquired diseases. For example, the *ras* oncogene mutations used in this study are found in a number of preneoplastic murine and human tumor models including those of skin, colon, and hematopoietic systems and are thought to be closely related with the early events in a tumor formation.[10] Therefore, a method for rapid and precise detection of such mutations has an extreme importance in both diagnosis and basic study of those diseases. Numerous methods are currently available for this purpose including DNA sequencing, probe hybridization,[11] single strand conformation polymorphisms,[12] and so forth. The present CAGE system has several advantages compared with those methods such as rapid measurement (~30 min), real time detection, and a small sample requirement (typically 10 ng).

REFERENCES

[1] Horejsi, V. and Ticha, M. (1986) *J. Chromatogr.*, 376, 49-67.

[2] Horejsi, V. and Ticha, M. (1981) *J. Chromatogr.*, 216, 43-62.

[3] Nakamura, K., Kuwahara, A. and Takeo, K. (1980) *J. Chromatogr.*, 196, 85-99.

[4] Tibanyenda, N., De Bruin, S. H., Haasnoot, C. A. G., Van der Marel, G. A., Van Boom, J. H. and Hilbers, C. W. (1984) *Eur. J. Biochem.*, 139, 19-27.

[5] Aboul-ela, F., Koh, D. and Tinoco, J., I. (1985) *Nucleic Acids Res.*,

13, 4811-4824.

[6] Werntges, H., Steger, G., Riesner, D. and Fritz, H.-J. (1986)
     Nucleic Acids Res., 14, 3773-3790.

[7] Ke, S.-H. and Wartell, R. M. (1993) *Nucleic Acids Res.*, 21, 5137-
     5143.

[8] Gotoh, O. and Tagashira, Y. (1981) *Biopolymers*, 20, 1033-1042.

[9] Akashi, M., Iwasaki, H., Miyauchi, N., Sato, T., Sunamoto, J. and
     Takemoto, K. (1989) *J. Bioact. Compt. Polym.*, 4, 124-136.

[10] Myers, R. M., Larin, Z. and Maniatis, T. (1985) *Science*, 230, 1242-
     1246.

[11] Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T.
     (1989) *Proc. Natl. Acad. Sci. USA*, 86, 2766-2770.

Part II

EFFECTS OF SYNTHETIC POLYANIONIC POLYMERS ON THE GROWTH OF CULTURED MAMMALIAN CELLS

INTRODUCTION

As already described in the General Introduction, Regelson's work presented a polyanionic polymer as a promising candidate of a novel biologically active polymer.[1,2] Since then, biological activities of various synthetic polyanionic polymers have been extensively examined. For example, aurintricarboxylic acid (Figure 1) showed inhibition of DNA and RNA polymerase reaction, anti-HIV activity and so forth.[3] A polyaromatic anionic compound, RG-13577, had antiproliferative activity to vascular smooth muscle cells.[4]

**Aurintricarboxylic acid (ATA)**　　　　　　　**RG-13577**
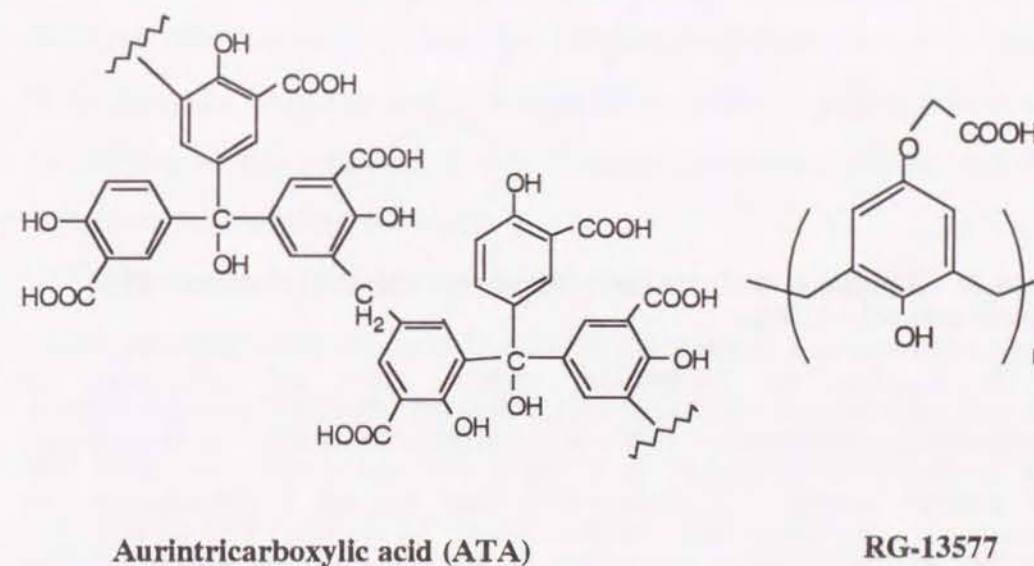
Figure 1. Proposed chemical structures of ATA and RG-13577.

Among the synthetic polyanionic polymers studied, maleic acid copolymers have unique characteristics; they have highly anionic alternate structures of a maleic acid unit and a comonomer unit.

65

Ottenbrite and his coworkers systematically investigated biological activity of maleic acid copolymers with respect to the chemical structure of side chain moieties, chain rigidity, hydrophobicity, and charge density.[5] From these studies, it was found that poly(maleic acid-alt-7,12-dioxaspiro[5.6]dodec-9-ene) (MA-CDA, Figure 2) has a variety of biological activities.[5] The activities include; activation of macrophages[6], induction of superoxide production by differentiated HL-60 cells,[7] and *ex vivo* induction of interleukin-6 and tumor necrosis factor-α in peripheral whole blood cells.[8,9]
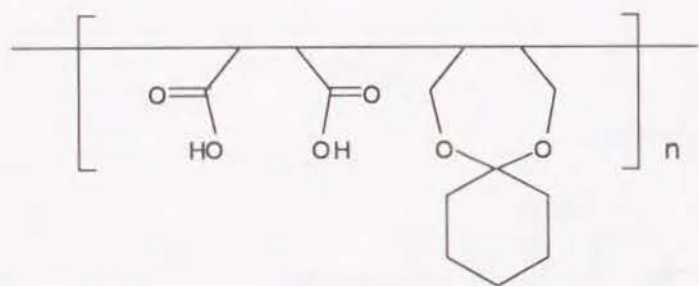
Figure 2. Chemical structure of poly(maleic acid-alt-7,12-dioxaspiro[5.6] dodec-9-ene) (MA-CDA).

A number of molecular events such as activation of phosphoinositide turnover, increase in intracellular Ca^{2+} concentration, and protein kinase C translocation from cytosol to membrane are observed in macrophage activation.[10] Recent studies on the cellular signal transduction system suggest that these events are also commonly associated with the signal transduction processes which occur in cell proliferation and differentiation.[11] In addition several macrophage activators are known to promote the growth of other cells.

For example, phorbol ester, a tumor promoter, exhibits both macrophage activation and growth stimulation through protein kinase C activation. These facts led the author to a hypothesis that, other than macrophage activation activity, a synthetic polyanionic polymer MA-CDA could also have a growth promotion activity to cells.

In Chapter 3, the effect of MA-CDA and other structurally related polymers on the growth of cultured mammalian cell lines is examined.

The action of a water soluble polyanionic polymer such as MA-CDA to cells begins from the interaction of the polymer with cell plasma membrane. The polyionic water soluble polymer is thought not to directly pass through lipid membrane into the interior of cells because the energetic barrier in the process must be quite large.[12] Therefore, the major modes of the interaction of the polymer with cells is binding of the polymer to cell plasma membrane and/or endocytic uptake of the polymer into cells.[13]

Naturally occurring growth factors are believed to have corresponding receptors on cell plasma membrane, and the binding of a growth factor induces the growth signal.[14] In the case of MA-CDA, it is not clear whether solely the binding of MA-CDA onto cell plasma membrane is sufficient for its growth promotion activity. Sunamoto and his coworkers reported that, in the macrophage activation by MA-CDA, encapsulation of the polymer into polysaccharide-coated liposome significantly enhanced the activity of MA-CDA.[15] The polysaccharide-coated liposome was subjected to higher uptake by macrophages compared to non-coated liposomes. These results suggest that the

enhanced activity of MA-CDA may be due to the enhanced uptake of the polymer, and that internalization of the polymer by macrophage should be essential to the macrophage activation.

Cultured fibroblasts can also uptake substances by endocytosis.[16] Therefore, MA-CDA may be internalized into L929 and STO cells during the incubation in the growth experiments. If this is the case, a possibility of the growth promotion of these fibroblasts by internalized MA-CDA may have to be considered.

In Chapter 4, the interaction of MA-CDA and other structurally related polyanionic polymers with cell plasma membrane is estimated using liposomal model membrane. As the first step of the elucidation of the action of MA-CDA to fibroblasts, internalization of MA-CDA by cultured fibroblasts is evaluated. The results are discussed in relation with the growth promotion activity of MA-CDA observed in Chapter 3.

REFERENCES

[2]   Feltz, E. T. and Regelson, W. (1962) *Nature*, 196, 642-645.

[3]   Cushman, M., Kanamathareddy, S., Clercq, E. D., Schols, D., Goldman, M. E. and Bowen, J. A. (1991) *J. Med. Chem.*, 34, 337-342.

[4]   Benezra, M., Ben-Sasson, S. A., Regan, J., Chang, M., Bar-Shavit, R. and Vlodavsky, I. (1994) *Arterioscler Thromb.*, 14, 1992-1999.

[5]   Ottenbrite, R. M., Kuus, K. and Kaplan, A. M. (1983) In *Polymers in Medicine*, 3-22, Plenum Pub. co., New York.

[6]   Ottenbrite, R. M., Sunamoto, J., Sato, T., Kojima, K., Sahara, K., Harat, K. and Oka, M. (1988) *J. Bioact. Compt. Polym.*, 3, 184-190.

[7]   Suda, Y., Kusumoto, S., Oku, N., Yamamoto, H., Sumi, M., Ito, F. and Ottenbrite, R. M. (1992) *J. Bioact. Compt. Polym.*, 7, 275-287.

[8]   Suda, Y., Kusumoto, S., Oku, N. and Ottenbrite, R. M. (1994) In *Polymeric Drugs and Drug Administration.* 149-162, ACS Symposium Series 545.

[9]   Suda, Y., Kusumoto, S., Oku, N., Ding, J.-L. and Ottenbrite, R. M. (1994) *Polymer Preprints* 415, 35,

[10] Rossi, F. (1986) *Biochim. Biophys. Acta*, 853, 65-89.

[11] Karin, M. (1992) *FASEB J.*, 6, 2581-2590.

[12] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (1994) In *Molecular Biology of the Cell*, Garland Publishing, Inc., New York and London.

[13] Noronha-Blob, L. and Pitha, J. (1978) *Biochim. Biophys. Acta*, 519, 285-290.

[14] Ullrich, A. and Schlessinger, J. (1990) *Cell*, 61, 203-212.

[15] Sato, T., Kojima, K., Ihda, T., Sunamoto, J. and Ottenbrite, R. M. (1986) *J. Bioact. Compt. Polym.*, 1, 448-460.

[16] Steinman, R. M., Silver, J. M. and Cohn, Z. A. (1974) *J. Cell Biol.*, 63, 949-969.

Chapter 3

Enhanced growth of cultured fibroblasts by
synthetic polyanionic polymers

As already explained, poly(maleic acid-alt-7,12-dioxaspiro-[5.6]dodec-9-ene) (MA-CDA) possesses a possibility to show growth promoting activity to cells other than macrophages. In the present study, the effect of MA-CDA and other structurally related polymers on mammalian cell growth was investigated. In the case of natural growth factors such as epidermal growth factor (EGF) contained in a serum, the action of a growth factor is known to be drastically influenced by the co-existing of other growth factors.[1] A famous example is transforming growth factor-β (TGF-β),[2] which stimulates growth of certain fibroblasts *in vitro* in the presence of platelet-derived growth factor (PDGF) but inhibits their growth if EGF is present.[2] Therefore, the effect of MA-CDA on cell growth was examined both with and without natural growth factors. In addition, it is also known that the action of growth promotive substances depend on cell type.[1] In the present study, the effect of MA-CDA was tested with several different mammalian cell types; L929 and STO mouse fibroblasts, B16 mouse melanoma, HeLa human carcinoma, and RPMI 4788 human colon cancer cells.

MATERIALS AND METHODS

Materials

All the polyanionic polymers except poly(maleic acid-alt-2-buten-1,4-diol) (MA-BD) were synthesized and purified by the laboratory of Professor R. M. Ottenbrite at Virginia Commonwealth University.[3]   Poly(maleic acid-alt-7,12-dioxaspiro[5.6]dodec-9-ene) (MA-CDA) with weight-average molecular weights of 1,500, 6,000, 10,000, and 22,000 were abbreviated to MA-CDA-1.5k, -6k, -10k, and -22k, respectively.   The ratio of weight-average molecular weight to number-average molecular weight was less than 1.5 for all the MA-CDAs.   Poly(maleic acid-alt-styrene) (MA-ST), poly(maleic acid-alt-4-methyl-4-penten-2-one) (MA-MP), and poly(maleic acid-alt-allylurea) (MA-AU) have average molecular weights of 20,000, 10,000, and 20,000, respectively.   MA-BD was obtained by acid hydrolysis of MA-CDA-30k.   All the structures of the polymers are shown in Figure 3-1.

Eagle's minimal essential medium (EMEM), Dulbecco's modified Eagle medium (DMEM), RPMI 1640 medium and sterilized glutamine were purchased from Nissui Pharmaceutical Co., LTD. (Tokyo). A culture flask (Cat. No. 25100-25), a 24-well microtiter plate (Cat. No. 25820-24), and a 96-well microtiter plate (Cat. No. 25860-96) were obtained from Corning Costar (Corning, NY) A fetal bovine serum (FBS) was obtained from Whittaker Bioproducts (Walkersville, MA) and heat-inactivated at 56 °C for 30 min before use. Heparin sodium salt (from porcine intestinal mucosa, grade I-A) and

transferrin (holo type) were purchased from Sigma (St. Louis, MO). Trypsin was obtained from DIFCO Laboratories (Detroit, MI). Insulin from bovine pancreas and other reagents were of the special grade and purchased from Wako Pure Chemicals Industries, LTD. (Osaka). MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-$2H$ tetrazolium bromide was obtained from Dojindo (Kumamoto).
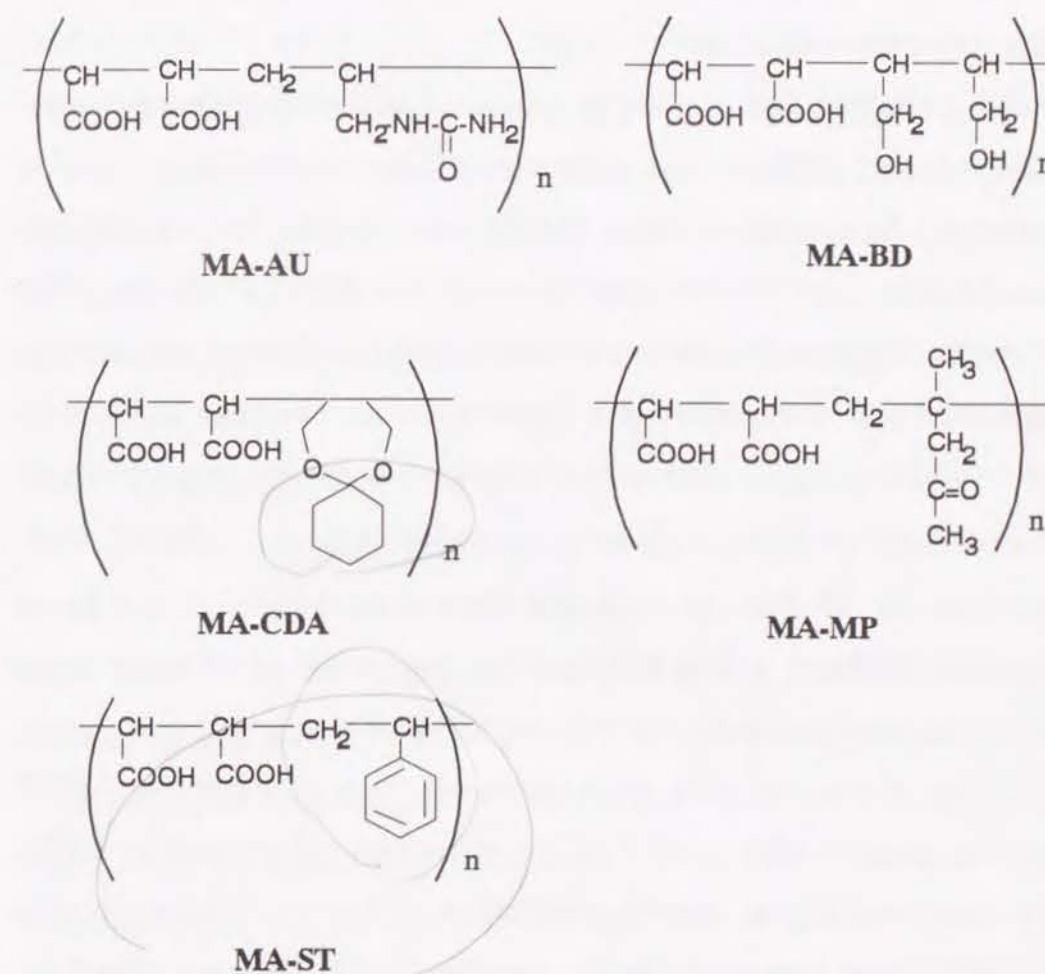

Figure 3-1. Structures of polyanionic polymers used in this experiment.

Growth assay

Before the experiments, the limulus activity of MA-CDA and heparin was checked by Endospecy® test (Seikagakukogyo, Tokyo). The level of endotoxin found in 100 μg/ml solutions of the respective polymers was less than 0.022 ng/ml for MA-CDA and 0.013 ng/ml for heparin. These values were far below the level at which endotoxin is known to show its biological activities such as macrophage activation[4] and mitogenesis induction.[5]

Cells were maintained in a basal medium supplemented with 10 % FBS and 0.03 % glutamine in culture flasks at 37 °C in a 5 % CO_2 incubator. As a basal medium, EMEM were used for HeLa and L929 cells, DMEM for STO cells, RPMI 1640 for RPMI 4788 and B16 melanoma cells. The cells in their growth phase were lightly trypsinized and resuspended in a basal medium containing 10 vol % of FBS to be 10^5 cells/ml. The cell suspension was seeded at a density of 2.5×10^4 cells/cm^2 on a 24-well microtiter plates. After 8 hour incubation at 37 °C, the cell monolayer was washed twice by a phosphate buffered saline (PBS). The medium was changed to a serum-free basal medium, and cells were further incubated for 16 hours at 37 °C. Then, the cells were incubated with a serum-free MEM containing a given amount of MA-CDA or heparin. A serum-free MEM alone was used for the control experiment. After the incubation, the cell proliferation was evaluated by counting living cells not stained by erythrosin B. The results are expressed as the relative ratio of the number of living cells to that in the control experiment. All the runs

were quadruplicated.

Growth promotion activity of a conditioned medium

A cell suspension obtained according to the method described above was seeded into 24-well microtitier plates at a density of 6.5 × 10^4 cells/cm^2. After 8 hour incubation at 37 °C, the cell monolayer was washed twice by a phosphate buffered saline (PBS). The medium was changed to a serum-free basal medium, and cells were further incubated for 16 hours at 37 °C. Then, the cells were incubated in a serum-free MEM or MEM containing 20 μg/ml of MA-CDA for 6 hours. A serum-free MEM without MA-CDA was used for the control experiment. After the incubation the medium was removed, and the cell monolayer was washed once with PBS. The cells were incubated in a fresh MEM. At 6, 12, and 24 hour intervals, the supernatant (the conditioned medium) was collected. The conditioned medium thus obtained was passed through a 0.2 μm pore filter to remove debris.

The growth promotion activity of the conditioned medium was determined by means of the conventional colorimetric assay using MTT tetrasolium. The cell suspension was seeded at a density of 2.5 × 10^4 cells/cm^2 on a 96-well microtiter plates. After 8 hour incubation at 37 °C, the medium was changed to a serum-free basal medium, and cells were further incubated for 16 hours at 37 °C. Then the cells were incubated with the conditioned medium for 48 hours. After the incubation, a stock solution of MTT (5 mg/ml in PBS) was added to all the wells (10 μl per 100 μl medium), and the plates were incubated at

37 °C for 3 hours. The blue crystals produced were dissolved by adding 100 μl of isopropanol, and the absorbance of the supernatant at 570 nm was measuring by a Model 450 microplate reader (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Effects of MA-CDA on the growth of cultured mammalian cells

Addition of MA-CDA alone to a serum- and protein-free basal medium caused growth enhancement of cultured cells, and the activity was specific with respect to the cell type (Figure 3-2). Among the cell lines examined, only L929 and STO mouse fibroblasts were responsive to the polymer, resulting in the proliferation of both fibroblasts. The proliferation of L929 and STO fibroblasts was dependent of the concentration of MA-CDA (Figure 3-3). For L929 cells, the highest growth promotion was obtained at 20 μg/ml of MA-CDA, resulting 1.9-fold higher growth of the cells than culture without MA-CDA. Above the concentration, the number of L929 cells decreased. In the case of STO cells, however, the cell number increased monotonously with increasing of the concentration of MA-CDA up to 100 μg/ml. At that concentration, the cell number was 3.7-fold higher than that without MA-CDA.

Figure 3-4 shows the effect of MA-CDAs with different molecular weights ranging from 1500 to 20000 on the growth of L929 cells. From this Figure, although all MA-CDAs promoted the growth
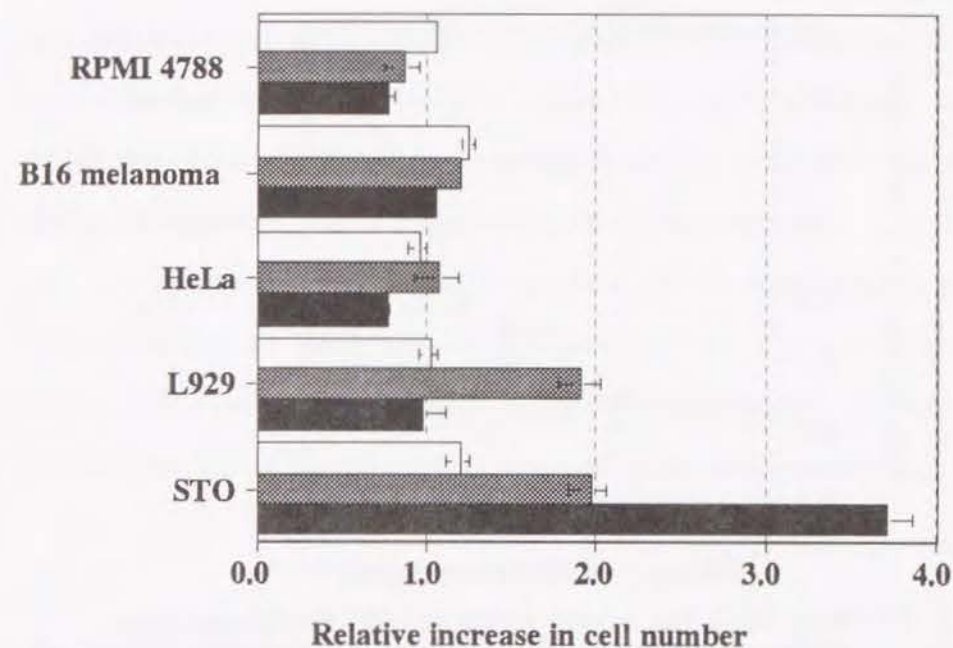
Figure 3-2. Effects of MA-CDA on the growth of various cell lines in a serum-free medium. Cell lines employed in this experiment were RPMI 4788 human colon cancer cell, B16 mouse melanoma, HeLa human carcinoma, L929 mouse fibroblasts, and STO mouse fibroblast. All the cell lines were incubated with 1 μg/ml (□), 10 μg/ml (▨) and 100 μg/ml (■) of MA-CDA for three days. The results are expressed as the relative ratio of the cell number with MA-CDA to that without MA-CDA.
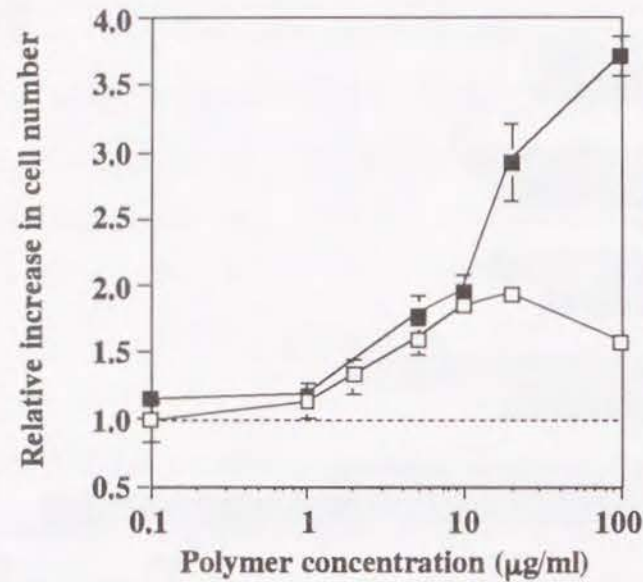
Figure 3-3. Effects of MA-CDA on the growth of L929 fibroblasts (open squire) and STO fibroblasts (closed squire) in a serum-free medium. Both cell lines were incubated with the indicated concentration of MA-CDA for 2 days.
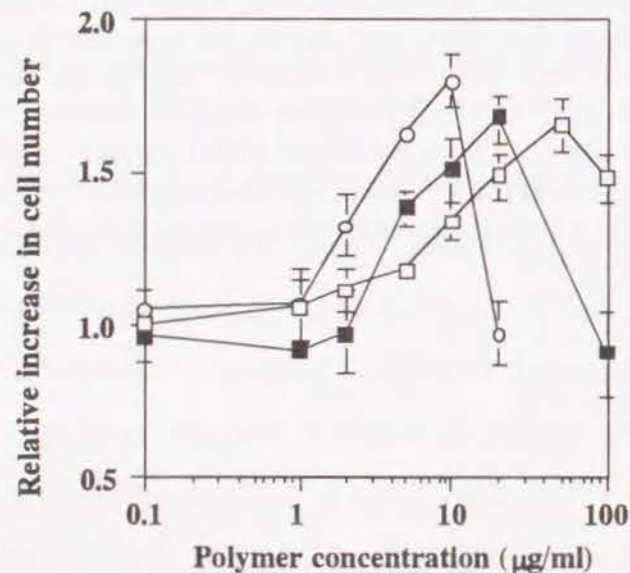

Figure 3-4. Effect of the molecular weight of MA-CDA on its growth promotion activity for L929 fibroblasts. Molecular weights of MA-CDA are 1,500 (□), 5,000 (■), and 22,000 (○). L929 cells were incubated with the indicated concentration of the polymer for two days.

of L929 cells, the concentration which effected the maximum promotion was different among MA-CDAs. MA-CDA with molecular weight of 1500 needed 5 times higher concentration than that with 22000 to show a similar extent of the growth promotion activity.

Figure 3-5 shows the changes in the number of L929 cells as a function of time. In a serum- and protein-free medium without MA-CDA, L929 cells were found to hardly proliferate. After 4 days incubation, the cells completely stopped their proliferation.
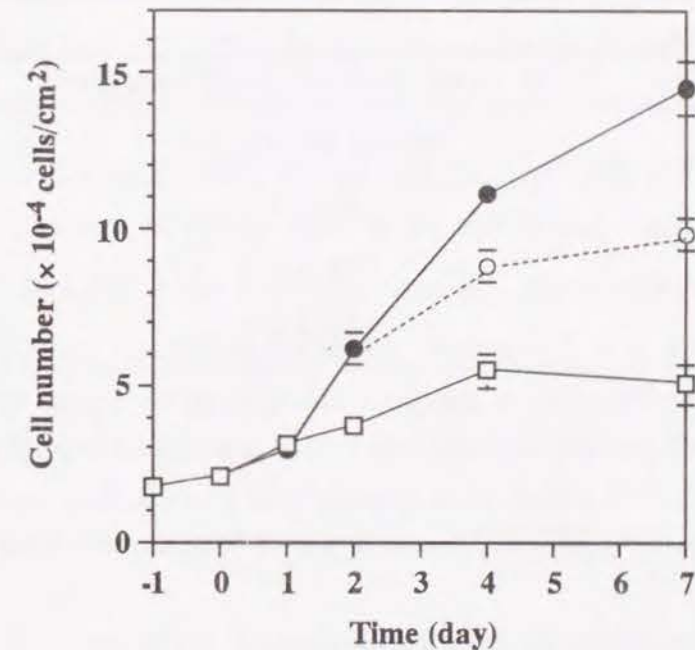

Figure 3-5. Growth profile of L929 fibroblasts with or without MA-CDA in a serum-free medium. L929 cells were cultured with 10 µg/ml of MA-CDA for 7 days (closed circle), with 10 µg/ml of MA-CDA for early 2 days and then with a basal medium (open circle with dashed line), and with a basal medium (open squire).

By addition of MA-CDA in the serum- and protein-free medium, L929 cells began to show active proliferation approximately

after 24 hours. This kind of induction for growth enhancement was not observed when a fetal bovine serum was used in place of MA-CDA (data not shown). The enhanced growth of L929 cells continued for at least 7 days. On the other hand, removal of the polymer from the medium reduced the proliferation of L929 cells to a level similar to that in a serum- and protein-free medium without the polymer. At 20 μg/ml of MA-CDA, exposure of L929 to MA-CDA for 3 h was sufficient to observe the full extent of the growth promotion activity (Table 3-1).

Table 3-1 Exposure period of L929 cells to MA-CDA and the growth promotion

Exposure period (h)	Relative increase in cell number to the control (%)
1	132 ± 13
3	158 ± 3.0
6	162 ± 1.0

L929 cells were incubated in a medium containing 20 μg/ml of MA-CDA for a prescribed period, washed twice with a phosphate buffer and cultivated further for 48 hours in a serum- and protein-free medium. In the control experiment, MA-CDA was omitted from the medium.

Growth promotion activity in a conditioned medium

A possible action of MA-CDA is to induce the production of a growth promoting factor by cell itself. To test this possibility, L929 cells were exposed to a conditioned medium which was collected from another culture of L929 cells preinteracted with MA-CDA. The cell growth in L929 cultures incubated with the conditioned medium was

identical with that in a comparable experiment without using MA-CDA. Relative cell numbers grown in the experiments with MA-CDA against that without MA-CDA were 105 ± 4 %, 99 ± 4 %, and 103 ± 5 % for the conditioned media sampled at 6, 12, and 24 hours of the preinteraction, respectively.

Effect of naturally occurring growth factors on the growth promotion activity of MA-CDA

The actions of naturally occurring growth factors are known to be influenced by coexisting other growth factors.[1] The effect of naturally occurring growth factors in a serum and/or insulin/transferrin, on the growth promotion activity of MA-CDA are summarized in Figure 3-6. Addition of these factors to the basal medium promoted the growth of L929 cells two-fold, however, the coaddition of MA-CDA to the medium with these growth factors did not further enhance the growth.
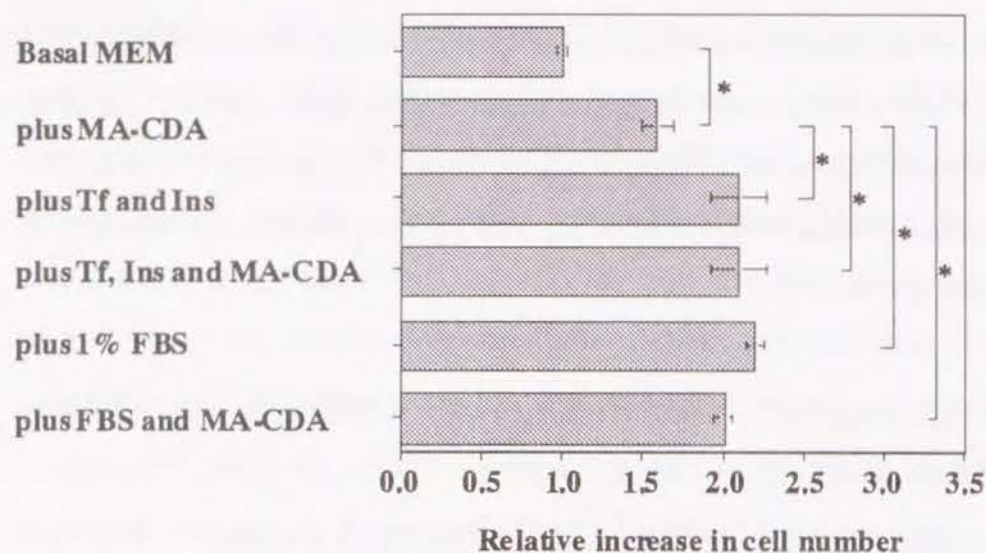
Figure 3-6. Effect of growth factors on growth promotion activity of MA-CDA. Cells were incubated for 2 days with various additives; 20 μg/ml of MA-CDA, 5 μg/ml of transferrin (Tf), 5 μg/ml of insulin (Ins), or 1 % (v/v) of fetal bovine serum (FBS). Asterisk (*) indicates statistically significant difference with p<0.005 as estimated by the Student t-test.

Growth promotion activity of polyanionic polymers structurally related to MA-CDA

Figure 3-7 shows the effect of various maleic acid copolymers including MA-CDA on the growth of L929 cells. Compared with MA-CDA, MA-ST showed weaken but significant growth promotion activity. MA-ST also promoted the growth of STO fibroblasts in a serum-free medium but did not promote the growth of HeLa cells (data not shown). On the other hand, only marginal effect of MA-BD, MA-AU, or MA-MP on the growth of L929 cells was observed.

For comparsison, growth promotion effect of a naturally occurring polyanionic polymer heparin was also examined. The result

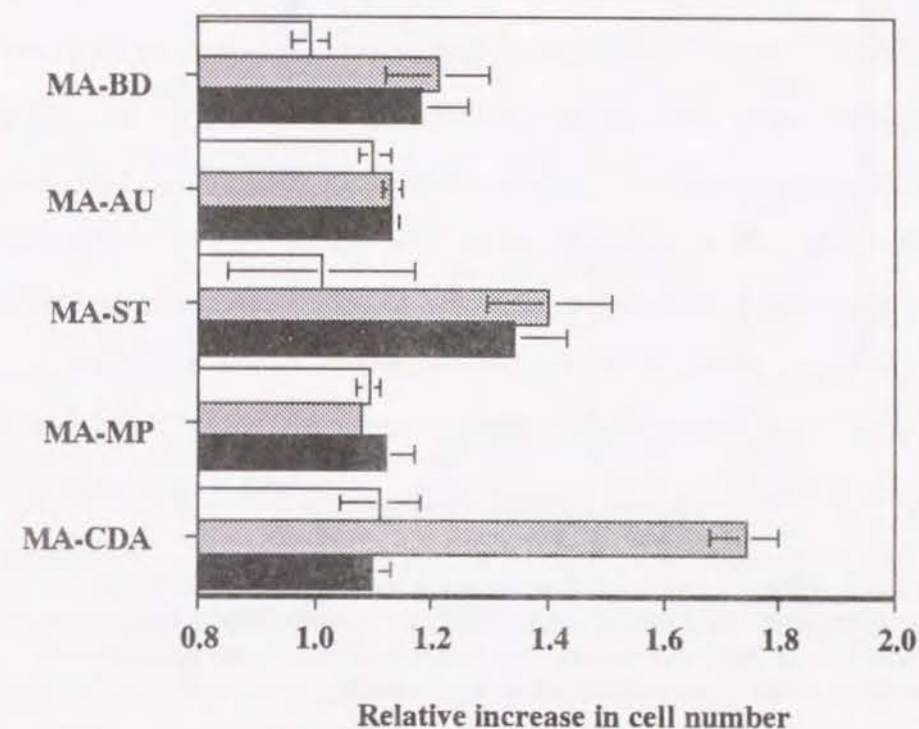in Figure 3-8 indicates that heparin has no growth promotion under the similar condition.

Figure 3-7. Effects of various maleic acid copolymers on the growth of L929 fibroblasts in a serum-free medium. L929 cells were incubated with 1 μg/ml (□), 10 μg/ml (□), and 100 μg/ml (■) of an indicated polymer for two days. The results are expressed as the relative ratio of the cell number in an experiment with a polymer to that without a polymer.
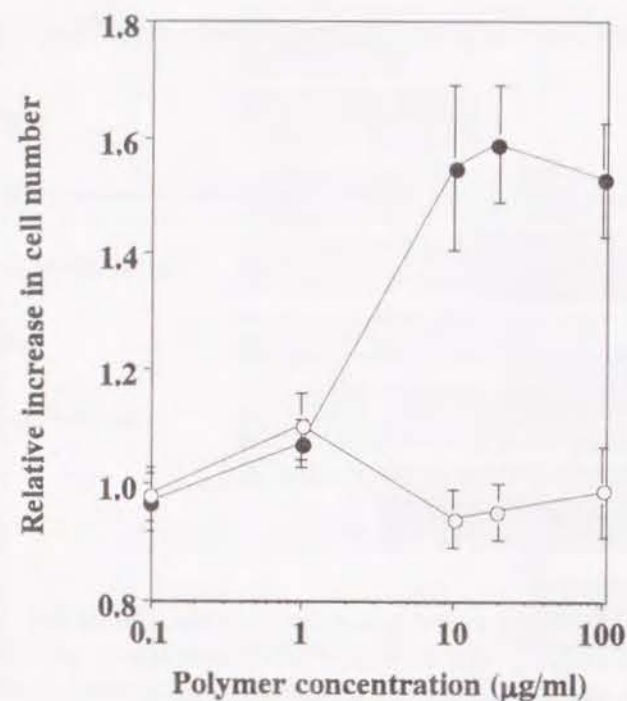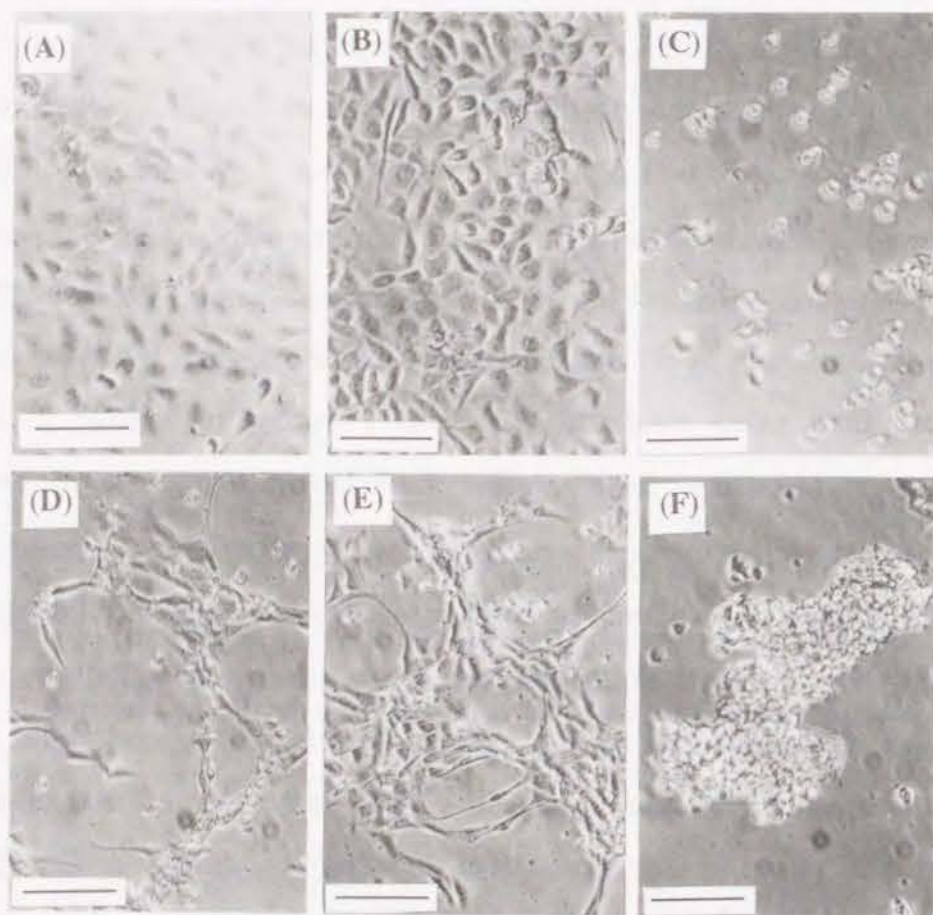
Figure 3-8. Effect of MA-CDA or heparin on the growth of L929 mouse fibroblasts as a function of polymer concentration. Cells were incubated for 2 days in a serum-free basal medium in the presence of MA-CDA (closed circle) or heparin (open circle).

Effects of polyanionic polymers on the morphology of cultured mammalian cells

Other than the growth promotion, addition of MA-CDA also induced the morphological changes of both L929 and STO fibroblasts at concentration of MA-CDA above 50 µg/ml. In Figure 3-9, microscopic images of L929 and STO fibroblasts at various polymer concentrations are shown. Both fibroblasts did not alter their morphologies at 10 µg/ml of MA-CDA (Figure 3-9B and E), which was MA-CDA concentration sufficient for the growth enhancement, from that without

the polymer (Figure 3-9A and D). At 100 µg/ml of MA-CDA, however, the polymer caused morphological changes in both L929 (Figure 3-9C) and STO (Figure 3-9F). L929 cells became swollen and partly detached from the culture plate in the presence of MA-CDA higher than 50 µg/ml (Figure 3-9C). After a while, the detached cells died, resulting in decrease of the cell number. On the other hand, STO cells formed spheroids under the same conditions (Figure 3-9F). This spheroid formation by STO cells was not observed when the cells were cultured in a same medium containing a serum regardless of the presence of MA-CDA (100 µg/ml). Cell detachment and spheroid formation are phenomena associated with a decrease of the interaction between a cell and an attaching substrate. It seems that at 100 µg/ml MA-CDA weakens the adhesion of the growth responsive cells to the substrate. However, the spheroid formation by STO cells under the conditions used indicates that MA-CDA may not affect cell-cell adhesion. A similar effect on cell morphology of L929 and STO was also observed for MA-ST. The morphological change only occurred on those cells that showed the growth promotion by MA-CDA or MA-ST. Furthermore, those polymers that failed to promote cell growth did not cause the morphological change at the concentration up to 500 µg/ml.
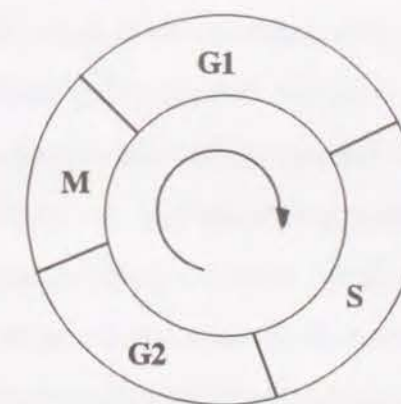
DISCUSSION

Cell proliferation proceeds along a certain cycle ("cell cycle"), consisting of four different phases; mitosis (M), gap 1 (G1), DNA synthesis (S), and gap 2 (G2) (Figure 3-10).[6] In order to move advance in a cell cycle, cells need to receive certain signals induced by stimulation from cellular surroundings at G1 phase. Without the signal, cells stop their proliferation and stay in G1 phase. Polypeptide growth factors such as platelet-derived growth factor (PDGF),[7] epidermal growth factor (EGF)[8] and insulin[9] are known as a potent inducer of the growth signal. These growth factors work on cells arrested at G1 phase and promote the growth of the cells.

Figure 3-9. Microscopic images of L929 fibroblasts (A)-(C) and STO fibroblasts (D)-(F) after 2 days incubation with or without MA-CDA. (A) and (D), control experiments without MA-CDA; (B) and (E), 10 µg/ml of MA-CDA; (C) and (F), 100 µg/ml of MA-CDA. (Bar = 100 µm).

Figure 3-10. Schematic representation of a cell cycle. M, mitosis; G1, gap 1; S, DNA sysnthesis; and G2, gap 2.

On the other hand, external factors can promote apparent growth of cultured cells by improving the survival of cells in cultivation.

For example carboxymethylcellulose shields L cells from possible damages during subculturing.[10] Naturally this type of growth promotion requires a coexisting growth signal inducer, and the effect is additive to that of the growth signal inducer.

In the present study, MA-CDA promoted proliferation of L929 cells growth-arrested in a serum-free medium without requiring any other external growth factors. Furthermore, the growth promotion effect was not additive to that of coexisting growth factors such as a serum and insulin. Therefore, the growth promotion effect of MA-CDA resembles that of a growth signal inducer.

There have been a few reports on growth promotion activities of synthetic polymers.[11-15] Katsuta and his coworkers observed a cell growth effect with poly(vinylpyrrolidone) for L929 cells in a serum-free medium.[11-13] However, this activity was more significant when a serum was present, which is not the case for MA-CDA. In addition, the concentration of poly(vinylpyrrolidone) had to be 100-times greater than that of MA-CDA in order to obtain a significant effect on cell proliferation. Taking into account subsequent reports,[10, 16, 17] poly(vinylpyrrolidone) seems to behave as an agent that protects the cytoplasm membrane during subculturing processes. Poly(ethylene oxide),[15] poly(vinyl-alcohol),[15] and pluronic polyols[14] promoted the growth of cultured cells. However these polymers required coexisting external growth factors in order to show their activities, which is also clearly different from the case of MA-CDA.

Heparin is a naturally occurring polyanionic polymer and known to stimulate the growth of various types of cells.[18, 19] Heparin is assumed to potentiate fibroblast growth factors, therefore, the presence of an external growth factor is essential.[20] Recently several synthetic polyanionic polymers such as poly(acrylic acid)[21] have also been reported for their heparin-like growth promoting activity. In the present study, heparin showed no effect on the growth of L929 cells under a comparable condition in which MA-CDA shows the growth promotion. This observation suggests that the mechanism of the cell proliferation by MA-CDA is different from that by heparin. Considering these differences, the direct growth promotion activity of MA-CDA found in this study is a unique and novel mode of biological activity a synthetic polymer can show.

Certain types of cells produce growth factors and respond to their own growth factors, which is termed autocrine secretion.[22] In addition a certain lectin such as concanavalin A is known to induce the secretion of growth factors from lymphocytes.[23] However a possibility that MA-CDA could promote cell proliferation by inducing the secretion of growth factors from cells was denied by the experiment showing no detectable activity of a conditioned medium collected from L929 cell culture preinteracted with MA-CDA. The action of MA-CDA is direct to L929 and STO fibroblasts.

The action of MA-CDA are also multifarious depending on cell type; triggering cytotoxic activity of macrophages,[3] induction of tumor necrosis factor (TNF) and interleukin-6 (IL-6) from blood cells,[24] and the growth promotion of fibroblasts observed in this study. All these cell

activities are known to involve signal transduction processes.[25] Although the mechanistic aspects of the biological activities of MA-CDA is yet to be elucidated, the present findings suggest that the action of the polymer involves the signal transduction pathway. The lack of growth promotion by MA-CDA in the presence of serum and/or insulin suggests that the growth promotion mechanism of MA-CDA should cause an interference to the promotion mechanism of those naturally occurring growth factors.

The present study also showed that the growth promotion effect of MA-CDA is transient. The removal of MA-CDA from the medium stops the growth promotion, thus MA-CDA does not cause permanent transformation of cells, which might accompany change of the proliferation behavior of cells.

The apparent correlation between the growth promotion effect and the change of cell morphology suggests that the interaction between cells and a polymer is only significant for certain pairs of a cell type and a polymer. For the first step for a polyanionic polymer to show its activity, interaction with the outer surface of plasma membrane must be important. This process probably depends on the physicochemical characteristics of the polymer such as hydrophilic-hydrophobic balance. Therefore the different growth promotion activity and effect on cell morphology observed among the polyanionic polymers examined may be explained by different binding of the polymers to lipid membrane. A study of the interaction of the polymers with model lipid membrane and actual cell plasma membrane is described in Chapter 4.

REFERENCES

[1] Sporn, M. B. and Roberts, A. B. (1988) *Nature,* 332, 217-219.

[2] Sporn, M. B. and Roberts, A. B. (1992) *J. Cell Biol.,* 119, 1017-1021.

[3] Ottenbrite, R. M., Kuus, K. and Kaplan, A. M. (1983) In *Polymers in Medicine,* 3-22, Plenum Pub. co., New York.

[4] Akagawa, K. and Tokunaga, T. (1985) *J. Exp. Med.,* 162, 1444-1459.

[5] Andersson, J., Melchers, F., Galanos, C. and Luderitz, O. (1973) *J. Exp. Med.,* 137, 943-953.

[6] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (1994) In *Molecular biology of the Cell,* 863-910, Garland Publishing, Inc., New York.

[7] Ross, R., Raines, E. W. and Bowen-Pope, D. F. (1986) *Cell,* 46, 155-169.

[8] Carpenter, G. and Cohen, S. (1990) *J. Biol. Chem.,* 14, 7709-7712.

[9] Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M. and Kahn, R. (1982) *Nature,* 298, 667-669.

[10] Thomas, J. A. and Johnson, M. J. (1967) *J. Natl. Cancer Inst.,* 39, 337-345.

[11] Katsuta, H., Takaoka, T., Mitamura, K., Kawada, I., Kuwabara, H. and Kuwabara, S. (1959) *Jap. J. Exp. Med.,* 29, 191-201.

[12]   Okumura, H., Takaoka, T. and Katsuta, H. (1960) *Jap. J. Exp. Med.*, 30, 159-170.

[13]   Kagawa, Y., Kaneko, K., Takaoka, T. and Katsuta, H. (1960) *Jap. J. Exp. Med.*, 30, 95-113.

[14]   Mizrahi, A. (1975) *J. Clin. Microbiol.*, 2, 11-13.

[15]   Shintani, Y., Iwamoto, K. and Kitano, K. (1988) *Appl. Microbiol. Biotechnol.*, 27, 533-537.

[16]   Bryant, J. C., Evans, V. J., Schilling, E. L. and Earle, W. R. (1961) *J. Natl. Cancer Inst.*, 26, 239-252.

[17]   Bryant, J. C. (1969) *Biotechnol. Bioengin.*, 11, 155-179.

[18]   Tardieu, M., Gamby, C., Avramoblou, T., Jozefonvicz, J. and Barritault, D. (1992) *J. Cell. Physiol.*, 150, 194-203.

[19]   Belford, D. A., Hendry, I. A. and Parish, C. R. (1992) *Biochemistry*, 31, 6498-6503.

[20]   Rosengart, T. K., Johnson, W. V., Friesel, R., Clark, R. and Maciag, T. (1988) *Biochem. Biophys. Res. Commun.*, 152, 432-440.

[21]   Hatanaka, K., Ohtsuki, T. and Kunou, M. (1994) *Chem. Lett.*, 1994, 1407-1410.

[22]   Sporn, M. B. and Roberts, A. B. (1985) *Nature*, 313, 745-747.

[23]   Taniguchi, T., Matsui, H., Fujita, T., Hatakeyama, M., Kashima, N., Fuse, A., Hamuro, J., Nishi-Takaoka, C. and Yamada, G. (1986) *Immunol. Rev.*, 92, 121-133.

[24]   Suda, Y., Kusumoto, S., Oku, N. and Ottenbrite, R. M. (1994) In *Polymeric Drugs and Drug Administration*   149-162, ACS Symposium Series 545, American Chemical Society.

[25]   Hirano, T. and Kishimoto, T. (1989) In *Handbook of Experimental Pharmacology*, M. B. Sporn and A. B. Roberts, Springer-Verlarg, Berlin.

Chapter 4

Binding and endocytosis as the primary interaction between polyanionic polymers and fibroblasts

In the previous chapter, two kinds of synthetic polyanionic polymers, poly(maleic acid-alt-7,12-dioxaspiro[5.6]dodec-9-ene) (MA-CDA) and poly(maleic acid-alt-styrene) (MA-ST), were found to promote the growth of cultured fibroblasts. Like naturally occurring growth factors, those polymers act directly on cells, and further, possibly on the signal transduction processes of cells.

In this chapter 4, this auther investigated the primary interaction between polyanionic polymers and cells, which occurs at the very early stage of the growth promotion. The most plausible primary phenomenon is binding and/or endocytosis between the polymers and the cells.

The growth promotion of fibroblasts was affected by both the chemical structure and the molecular weight of polyanionic polymers. The interaction of these polymers with lipid membrane also was evaluated by perturbation of liposomal membrane with the polymers. This gave an index of the association of the polymers to the lipid membrane. Furthermore, in order to investigate the actual uptake of MA-CDA by cells, MA-CDA was labeled with a fluorescent probe.

Intracellular distribution of the fluorescence labeled MA-CDA was photometrically and microscopically studied. These results are discussed with particular attention to the cell growth promotion activity of MA-CDA.

MATERIALS AND METHODS

Materials

Egg yolk lecithin (Egg PC) was obtained from Nippon Oil & Fats Co. (Tokyo). Cholesterol was of the special grade from Wako Pure Chemicals. Concentration of liposomal phosphatidylcholines was determined using a Phospholipid C-Test Wako kit of Wako Pure Chemicals (Osaka). 5(6)-Carboxyfluorescein (CF) was purchased from Eastman Kodak Company (Rochester, NY). All polyanionic polymers are the same those previously described in Chapter 3. 5-(Aminoacetamido)fluorescein was obtained from Molecular Probes, Inc. (Eugene, OR). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was purchased from Dojindo (Kumamoto).

Eagle's minimal essential medium (MEM, powder) and glutamine were commercially available from Nissui Pharmaceutical Co., LTD. (Tokyo). Fetal bovine serum (FBS) was from Whittaker Bioproducts (Walkersville, MA) and heat-inactivated at 56 °C for 30 min before use. A culture flask (Cat. No. MS-21250) was purchased from Sumitomo Bakelite Co., LTD. (Tokyo). A 24-well microtiter plate (Cat. No. 25820-24), and a 96-well microtiter plate (Cat. No. 25860-96) were

obtained from Corning Costar (Corning, NY). (4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were from Dojindo. Fluorescein isothiocyanate (FITC)-dextran that has average molecular weight of 19,600 was obtained from Sigma (St. Louis, MO). Other chemicals were purchased from Wako Pure Chemicals and used without further purification.

Release of CF from liposome induced by polyanionic polymers

CF-loading liposomes were prepared by the reversed evaporation method.[1] Egg PC (26.2 mg, approx. 3.4×10^{-5} mol) and cholesterol (3.3 mg, 8.3×10^{-6} mol) were dissolved in 1.0 ml of a mixed solvent (diethylether/dichloromethane = 6/4 by volume). To the lipid solution was added 0.4 ml of a concentrated CF solution (100 mM in PBS). The resultant mixture was sonicated by using a UD-201 cup-type ultrasonic disrupter (Tomy Seiko, Tokyo) for 30 sec. The lipid suspension thus obtained was transferred into a 10 ml round-bottom flask, and the organic solvent was removed by evaporating under reduced pressure (at 350 mmHg) for approximately 20 min at room temperature to form a gel. After the evaporation, 1.6 ml of PBS was added to the flask, and the contents were voltexed for 1 min to obtain a homogeneous liposomal suspension.

The liposomal suspension thus obtained was extruded (The Extruder, Lipid Biomembrane Inc. Vancouver, Canada) through a polycarbonate membrane (200 nm pore, Costar, Cambridge, MA) three

times. To remove an unencapsulated CF, the suspension (2 ml) was applied to a Sepharose 4B column (ϕ1.5 × 42 cm) as equilibrated with PBS. An eluted solution was fractionated by 120 drops each, and the absorbance of each fraction was measured at 496 nm by a U-3400 spectrophotometer (Hitachi, Tokyo). The fractions 6 and 7 contained the CF encapsulating liposome and used for further studies.

Phospholipid concentration of the liposomal suspension was adjusted to 2.0×10^{-5} M with PBS. To 0.5 ml of the suspension was added 0.5 ml of the PBS solution containing a polyanionic polymer, and the mixture was incubated for 15 h at 37 °C. After the incubation, the fluorescence intensity was measured at 516 nm on a 650-10S fluorospectrophotometer (Hitachi, Tokyo) with band pass of 5 nm. Then 20 µl of a 10 % aqueous solution of Triton X-100 was added to the suspension to destract liposomes and release CF completely. The fluorescence intensity of the solution gives the total concentration of CF in the initial liposomes. The amount of CF released was estimated according to the following equation:

$$\%\text{release} = \frac{I_t - I_0}{I_{Triton} - I_0} \times 100$$

where I_0 is the fluorescence intensity of a liposome suspension just after addition of the polymer, I_t is that after incubation at time t, and I_{Triton} is that after addition of the Triton X-100 solution.

Synthesis of MA-CDA labeled with fluorescein (fluorescein MA-CDA)

MA-CDA-16k, which has weight averaged molecular weight of 16,000, was covalently conjugated with fluorescein by amide bonds using the carbodiimide method.[2] MA-CDA-16k (32.8 mg) was dissolved in 15 ml of Milli-Q water and mixed with 5-(aminoacetamido)fluorescein (2.0 mg) dissolved in 0.5 ml of 0.1 N aqueous NaOH. After pH of the solution was adjusted to 5.0 with a 1.0 N aqueous HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (80.6 mg) was added to the solution, and the reaction mixture was stirred at room temperature for 1 h. Then the pH of the resultant solution was set to 9.8 with a 0.5 wt % aqueous $NaHCO_3$, and the solution was stirred at 40 °C for 2 h. The resulting solution was dialyzed 4 times against 3 liter of Milli-Q water for 2 days and lyophilized to obtain a yellow powder. Absence of unreacted 5-(aminoacetamido)fluorescein in the solid was confirmed using size exclusion chromatography. The amount of the fluorescein residues conjugated to MA-CDA was determined from the absorbance at 489 nm, which was estimated by an aqueous polymer solution using the molar absorption coefficient of fluorescein (6.5×10^4 mol·l⁻¹·cm⁻¹).[3]

L929 Cell culture

L929 mouse fibroblasts were maintained as monolayers on a culture flask in a MEM supplemented with 10 % FBS and 0.03 % glutamine at 37 °C in a 5 % CO_2 incubator. L929 cells were subcultivated by trypsinization before becoming confluent.

Growth promoting activity of fluorescein labeled MA-CDA

L929 cells in their growth phase were lightly trypsinized and resuspended to be 10^5 cells/ml. The cell was seeded at a density of 2.5 $\times 10^4$ cells/cm^2 on a 96-well microtiter plate. After incubation for 8 hr at 37 °C, the medium was changed to a serum-free basal medium, and the cells were incubated for another 16 hr at 37 °C. Then, the cells were incubated with a serum-free MEM containing a given amount of MA-CDA or fluorescein MA-CDA. A serum-free MEM without MA-CDA was used for the control. The cell proliferation was evaluated by a colorimetric assay using WST-1 and methoxy PMS[4] at 415 nm on a Model 450 microplate reader (Bio-Rad, Richmond, CA). The relative ratio of the number of living cells with MA-CDA to that without MA-CDA. All the runs were repeated four times.

Uptake of fluorescein MA-CDA or FITC-dextran by L929 cells

The cells in their growth phase were lightly trypsinized and resuspended to be 2×10^5 cells/ml in a basal medium containing 10 vol % of FBS. The cell was seeded at a density of 10^5 cells/cm^2 on a 24-well microtiter plate. After 8 hr incubation at 37 °C, the cell monolayer was washed twice by PBS. The medium was replaced by a serum-free basal medium, and cells were incubated for another 16 hrs at 37 °C. Then the monolayer of L929 cells was incubated at 37 °C in a serum-free basal medium supplemented with an appropriate amount of either FITC-dextran or fluorescein MA-CDA. After various periods

of incubation at 4 or 37 °C, the cell monolayer was rinsed three times with PBS, and then the cells were harvested by trypsinization and centrifuged. The cell pellets thus obtained were treated with 1 ml of a 1 % aqueous SDS solution. Fluorescence intensity of the cell lysate thus obtained was measured at 520 nm (excited at 496 nm) on a 650-10S fluorescence spectrophotometer.

Effect of a metabolic inhibitor on fluorescein MA-CDA or FITC-dextran uptake

The L929 cell monolayers were obtained on a 24-well plate as described above. The culture medium was replaced by 0.2 ml of MEM or MEM containing sodium azide (5 mM), and the cells were incubated for 1hr. Then 0.2 ml of MEM supplemented with either fluorescein MA-CDA or FITC-dextran were added to the well, and the cells were incubated for 1hr at 37 °C. After the incubation, fluorescein MA-CDA or FITC-dextran uptake was measured as described above.

Fluorescence microscopic observation

L929 cells were plated on a ZOG-3 small cell observation chamber (Elecon Kagaku, Chiba). The cell monolayers were incubated at 37 °C in a serum-free basal medium supplemented with FITC-dextran or fluorescein MA-CDA. Phase contrast and fluorescence images of cells were obtained with a MRC-600 confocal laser fluorescence microscope system (BioRad, Richmond, CA). The cells incubated with the polymers were excited at 488 nm using an Argon-ion laser, and the

fluorescence emission was monitored at higher wavelength above 515 nm.

RESULTS

Interaction of polyanionic polymers with liposomal membrane

Perturbation to liposomal membrane by polyanionic polymers was investigated by monitoring a release of 5(6)-carboxyfluorescein (CF) as encapsulated in liposome. Figure 4-1 indicates that the extent of perturbation was significantly affected by the polymer examined. MA-CDA and MA-ST caused a significant release of CF from the liposome depending on the polymer concentration. In addition, MA-CDA with higher molecular weight induced a complete release of the dye even at the lower polymer concentration compared with smaller MA-CDA. On the other hand, MA-MP and MA-BD did not induce the CF release at all within 15 hr of the polymer concentration up to 1mg/ml. It was coincident with the prvious result that MA-AU does not perturb liposomal membrane.[5]

Synthesis of fluorescein MA-CDA and its growth promotion activity

MA-CDA was labeled with an amino derivative of fluorescein using water-soluble carbodiimide as the condensation agent. Formation of amide bonds was confirmed by infrared spectroscopy. Figure 4-2 shows a structure expected for the fluorescein MA-CDA thus

prepared. Based on the fluorescence intensity of fluorescein MA-CDA, the amount of the fluorescein residue conjugated was found to be 0.83 per MA-CDA of the molecular weight 16,000.
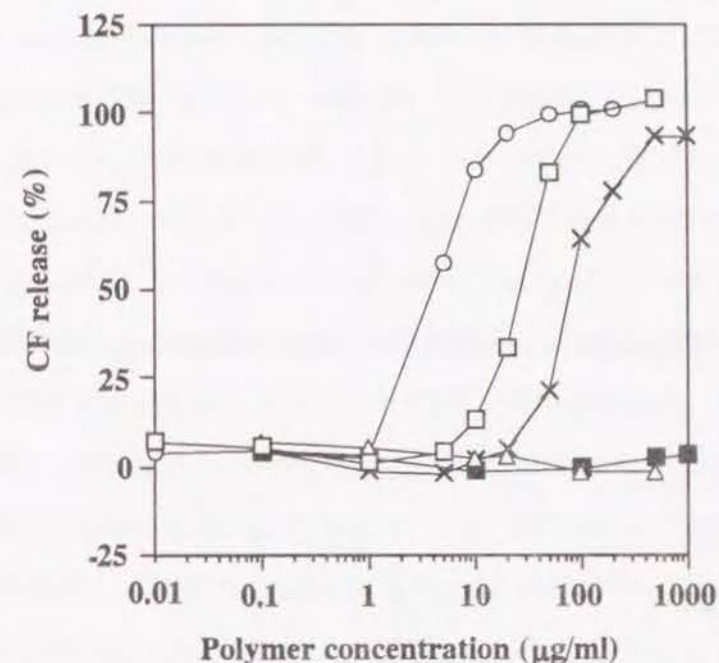
Figure 4-1. Carboxyfluorescein release from liposome by polyanionic polymers.
Polyanionic polymers; MA-CDA-1.5k (×), MA-CDA-22k (○), MA-ST (□), MA-MP(△), and MA-BD (■). Phospholipid concentration was 1.0×10^{-5} M. Incubation period: 15 hr. Temperature: 37 °C.

Effect of the fluorescein labeling on the growth promotion activity of MA-CDA was studied using L929 fibroblasts. Both labeled and unlabeled MA-CDA showed the same extent of the activity (Figure 4-3). In addition, the activity-concentration relationship of the two MA-CDAs was same (Figure 4-3). This meant that the effect of the labeling was negligible on the cell growth promotion activity of MA-
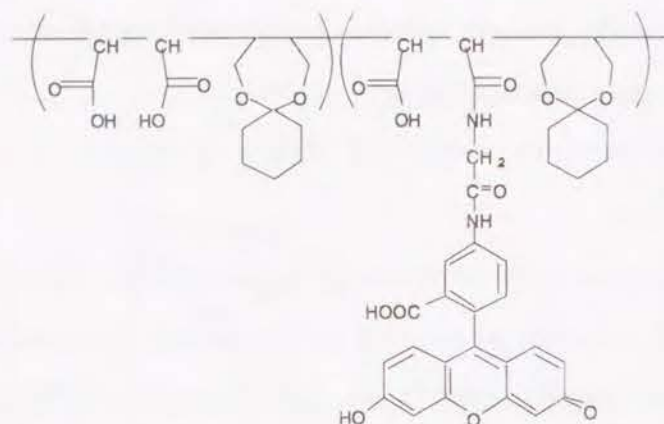
CDA.

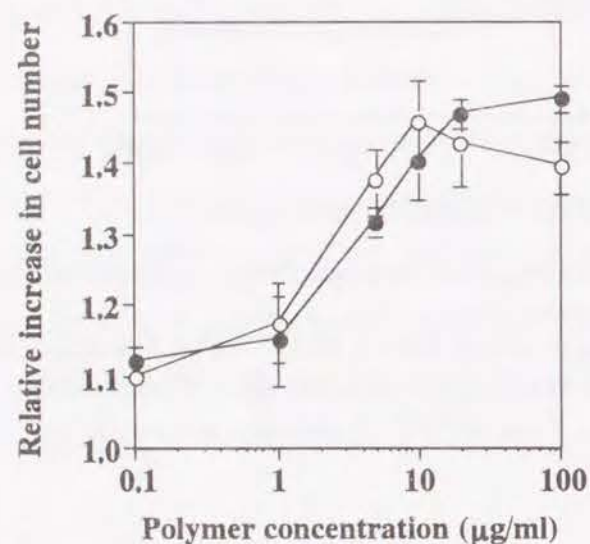Figure 4-2. Expected structure of fluorescein MA-CDA.

Figure 4-3. Growth promotion activity of unlabeled (open circle) and fluorescein labeled(closed circle) MA-CDA to L929 fibroblasts in a serum-free medium. L929 cells were incubated with the polymers for 2 days.

Uptake of fluorescein MA-CDA by L929 fibroblasts

L929 uptake of fluorescein MA-CDA was studied under the same serum-free condition as that used the growth promotion activity study.

Figure 4-4 shows the relation between the incubation time and the cellular uptake of the polymers. Because FITC-dextran is introduced by endocytosis[6] and does not show any physiological activity, it was used as the reference of the endocytic activity of the cell. At 37 °C, fluorescein MA-CDA as well as FITC-dextran was uptaken by L929. The uptake of fluorescein MA-CDA gradually decreased during the incubation (Figure 4-4A). However, FITC-dextran was introduced at a constant rate under the same condition, and a decrease in the cellular uptake was not observed different from the case of fluorescein MA-CDA. For both fluorescein MA-CDA and FITC-dextran, the amount of cell uptake increased as the polymer concentration in the medium became higher (Figure 4-5). However, the uptake of fluorescein MA-CDA levelled out at the higher polymer concentration. With FITC-dextran, no saturation was seen up to 2000 µg/ml When the uptake efficiency is defined by the % ratio of the amount of polymer uptaken to that administered per 10^5 cells (below 500 µg/ml), the uptake efficiency of fluorescein MA-CDA was much higher than that of FITC-dextran. For example, at 100 µg/ml of the polymer, the uptake efficiency was 0.22 % for fluorescein MA-CDA, while 0.0016 % for FITC-dextran.

The cell uptake drastically decreased at the low temperature (4 °C) for both fluorescein MA-CDA and FITC-dextran (Figure 4-4 and 4-5). However, small but obvious cell uptake was still observed with fluorescein MA-CDA, while the uptake of FITC-dextran was negligibly small. For both polymers, sodium azide, a metabolic inhibitor,[7] also lowered the cell uptake efficiency by approximately 40 % (Table 4-1).
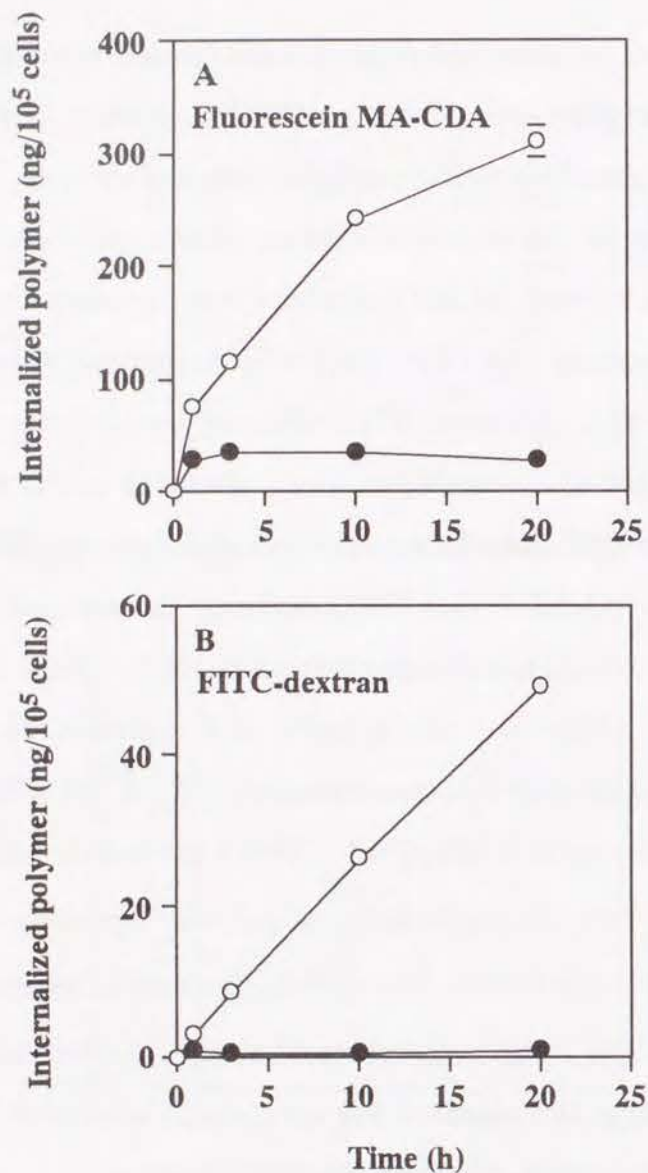
Figure 4-4. Cellular uptake of fluorescein MA-CDA (A) and FITC-dextran (B) by L929 fibroblasts at 4 °C (closed circle) and 37 °C (open circle). Concentration: 100 μg/ml of fluorescein MA-CDA and 1 mg/ml of FITC-dextran.
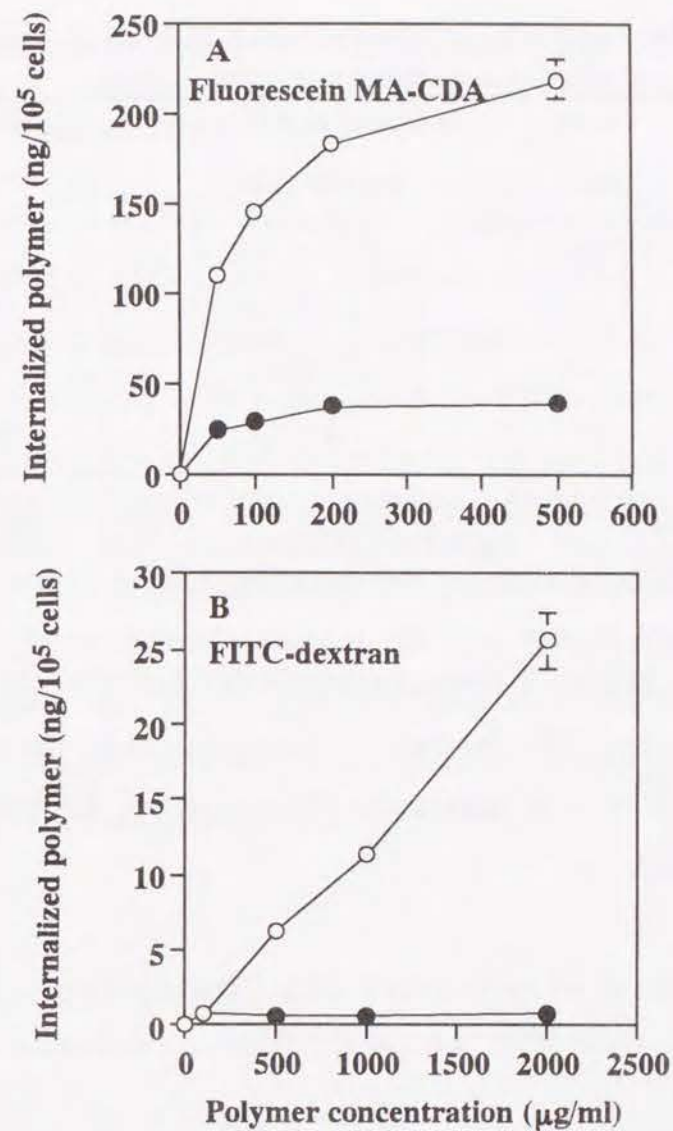
Figure 4-5. Cellular upatake of fluorescein MA-CDA (A) and FITC-dextran (B) by L929 fibroblasts at 4 °C (closed circle) and 37 °C (open circle) as a function of polymer concentration. Incubation time: 5 hr.

Table 4-1 Effect of NaN$_3$ on fluorescein MA-CDA uptake or FITC-dextran uptake by L929 mouse fibroblasts

	NaN$_3$ (mM)	Internalized polymer (ng/10^5 cells)	Inhibition (%)
MA-CDA	none	29.2 ± 3.4	
	5	11.4 ± 1.8	61
FITC-dextran	none	3.6 ± 0.2	
	5	1.6 ± 0.1	56

All the runs were triplicated. Polymer concentration: 100 μg/ml of fluorescein MA-CDA and 1 mg/ml of FITC-dextran. Incubation time: 1h. For the condition see Materials and Methods.

As shown in Table 4-2, the addition of unlabeled MA-CDA slightly reduced the uptake of FITC-dextran. On the other hand, the uptake of fluorescein MA-CDA was drastically decreased by the addition of unlabeled MA-CDA.

Table 4-2 Effects of unlabeled MA-CDA on the uptake of fluorescein MA-CDA and FITC-dextran

| Unlabeled MA-CDA added (μg/ml)	Uptake (ng/10^5 cells)	
	Fluorescein MA-CDA	FITC-dextran
none	552 ± 23 (100)	38 ± 0.3 (100)
100	101 ± 4.1 (18)	30 ± 1.4 (79)
500	51 ± 0.0 (9)	29 ± 1.6 (76)

Concentrations: 100 μg/ml of fluorescein MA-CDA and 1 mg/ml of FITC-dextran. L929 cells were incubated with fluorescein MA-CDA or

FITC-dextran in the presence of unlabeled MA-CDA for 5h at 37 °C. Values in parenthesis indicate the percent to the experiment without unlabeled MA-CDA.

Subcellular distribution of fluorescein MA-CDA

Subcellular distribution of fluorescein MA-CDA and FITC-dextran was investigated using a confocal laser microscope. Figure 4-6 shows both phase contrast and fluorescence images of L929 cells under the incubation for 1 hr at 37 °C with FITC-dextran (Figures 4-6 A and B) or fluorescein MA-CDA (Figures 4-6 C and D). FITC-dextran as the marker of endocytosis showed dot like distribution inside the cells (Figure 4-6 B). Fluorescein MA-CDA also showed similar dot like distribution in the cells. These results indicate that fluorescein MA-CDA was certainly endocytosed by L929 cells.
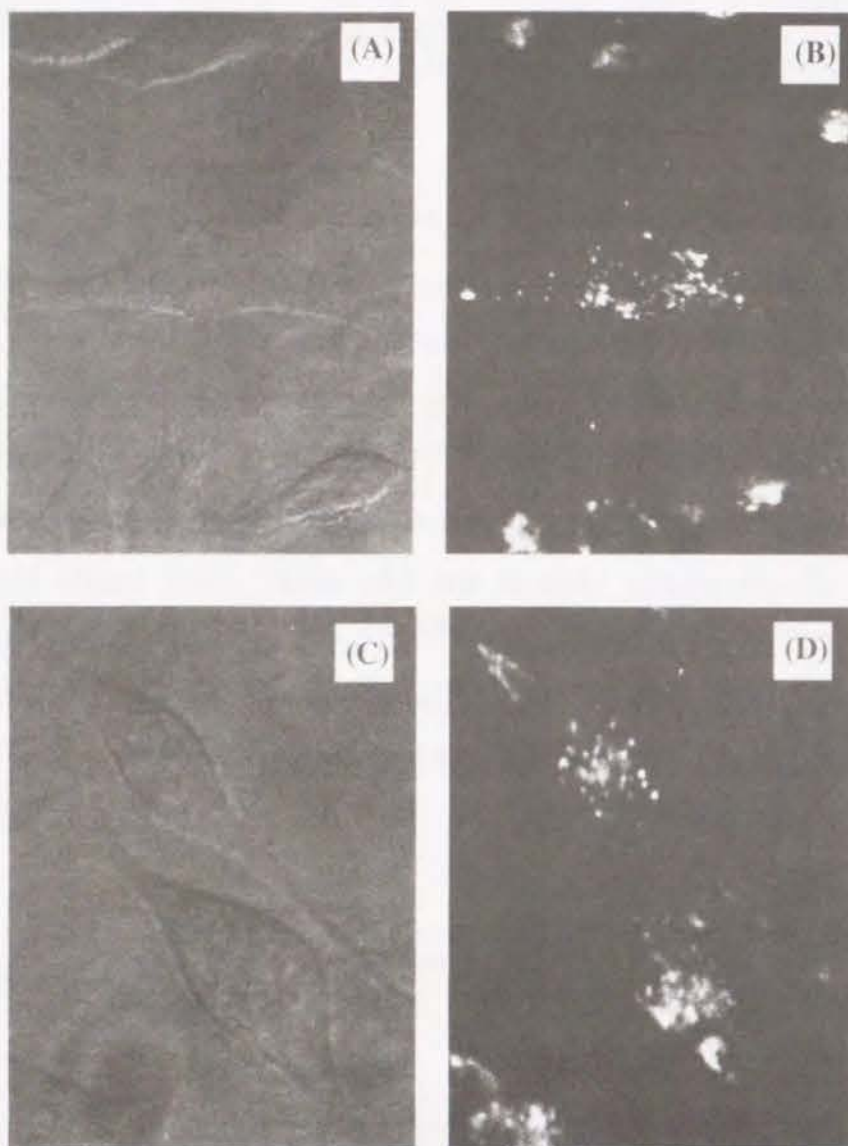
Figure 4-6. Microscopic images of L929 fibroblasts treated with FITC-dextran (A), (B) and fluorescein MA-CDA (C), (D). Phase contrast images: (A) and (C). Fluorescence images: (B) and (D). Concentration: 1mg/ml of FITC-dextran and 100 μg/ml of fluorescein MA-CDA. Incubation time: 1h.

DISCUSSION

Among the polyanionic polymers studied, the polymers those show the cell growth promotion activity showed also strong binding to lipid membranes. The extent of perturbation of the polyanionic polymers to liposomal membrane showed a good correlation with the growth promotion activity of the same polymers (see Chapter 3). MA-CDA and MA-ST, both of which were effective in the cell growth promotion, showed also significant perturbation to liposomal membrane. However, MA-BD and MA-MP, neither of which were active, did not show any efficiency in the cell growth promotion. Higher molecular weight MA-CDA showed both the higher growth promotion activity and the larger membrane perturbation.

Considering the chemical structure of the polymers in this study, the cyclohexylidene moiety of MA-CDA and the phenyl moiety of MA-ST should make the two polymers more hydrophobic than both of MA-BD and MA-MP. The present result agrees with previous studies, which suggest that the extent of liposomal membrane perturbation by the polyanionic polymers should increase for more hydrophobic polymers.[8-10] The dependence of cell growth promotion activity on the structure of maleic acid copolymers, therefore, can be ascribed to the hydrophobicity of the polymers.

In previous studies of biological activities of maleic acid copolymers such as macrophage activation and cytokine induction, the hydrophobicity of the polymer was also found to be important for those

biological activities.[9,11] Among those, a similar correlation between the perturbation to liposomal membrane and a biological activity of maleic acid copolymers has been reported for macrophage activation. Suda and his coworkers examined maleic acid copolymers, which showed macrophage activation, relating with the ability to perturb liposomal membrane. They found a positive correlation between the extent of perturbation and the macrophage activation efficiency.

The study further estimated the binding of those polymers to intact cells. The binding was also positively correlated with the perturbation.[8] This correlation also has been explained by hydrophobicity of the polymers. Presumably, the hydrophobic part of the polymer inserts itself into the liposomal membrane to cause the perturbation. Similarly, the part associates with hydrophobic domain of cell plasma membrane resulting in the binding to cells. The polyanionic nature of maleic acid copolymers is expect to prevent these polymers from interacting with cell plasma membrane negatively charged.[12] It is conceivable that an extra factor such as hydrophobic association should be necessary for the polymers to interact with cell membranes.

Considering these facts, the difference in the primary structure among the maleic acid copolymers should be reflected on the hydrophobicity of the polymers and affect their binding to the cells. The difference in the cell growth promotion activity of the polyanionic polymers, therefore, may be attributed to the different binding of the polymers to cell plasma membrane.

For maleic acid copolymers, relation between the membrane perturbation and the biological activities seems to be valid. For polyanionic polymers other than maleic acid copolymers, however, an ability to cause the liposomal membrane perturbation may not necessarily mean that a polymer has the growth promotion activity to fibroblasts. For example, poly(styrene sulfonate) caused the CF release, although this polymer rather showed cytotoxicity to fibroblasts (data not shown).

If MA-CDA and other maleic acid copolymers induce the cell growth promotion by acting extracellularly, the association of these polymers with cells would control the activity. On the other hand, it is also plausible that the polymers induce the cell growth promotion after internalization into the cells.

No difference in the growth promotion activity between fluorescein MA-CDA and unlabeled MA-CDA indicates that the interaction of MA-CDA with L929 cells is not significantly affected by the fluorescein labeling. Therefore the results obtained in the present study for fluorescein MA-CDA should be a good description of the interaction of MA-CDA with L929 cells.

A significant part of MA-CDA would be internalized into cell *via* endocytosis, because the uptake of fluorescein MA-CDA salso was by endocytosis.[7] The uptake was suppressed at lower temperature (4 °C) and by a metabolic inhibitor, sodium azide.[10] Results of the microscopic observation are also consistent with the endocytic internalization of MA-CDA.

The suppression of cell uptake in the presence of unlabeled MA-CDA and the saturation phenomenon of the uptake as a function of fluorescein MA-CDA concentration indicate the rate of cell uptake should be controled by the number of binding site. In addition, the significant inhibition of cell uptake with the pretreatment of unlabeled MA-CDA strongly suggests that the cell uptake would be receptor-mediated uptake. A large difference in the uptake efficiency observed between fluorescein MA-CDA and FITC-dextran is explained by binding specificity to cell plasma membrane between the two. This is suggested also from nonspecific but strong association of MA-CDA to liposomal membrane. Highly hydrophilic FITC-dextran does not bind much to the cell plasma membrane. Enhancement of the uptake activity of cells by MA-CDA is unlikely, because the uptake of FITC-dextran also decreased by addition of unlabeled MA-CDA.

The endocytic activity of cell is known to depend on the cell state including cell metabolism[7] and cell cycle.[6] The gradual decrease of the fluorescein MA-CDA uptake after long term incubation and the moderate suppression of the FITC-dextran uptake by unlabeled MA-CDA suggest that the interaction of L929 cells with MA-CDA should suppress the receptor-mediated endocytosis of the cells. This suppression, as well as the growth promotion, might be certain change in the cell state as induced by the interaction with MA-CDA.

At present, a problem still remains whether the binding of the polymer to the cell plasma membrane could solely induce the growth promotion through a signal transduction or the polymer would

intracellularly affect the the growth. In a previous study regarding macrophage activation by maleic acid copolymers, the polymers were introduced specifically to the inside of the cells using endocytic uptake of the polymer-loading liposome.[5].

REFERENCES

[1] Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E. and Papahadjopoulos, D. (1980) *Biochim. Biophys. Acta*, 601, 559-571.

[2] Nakajima, N. and Ikada, Y. (1995) *Bioconjugate Chem.*, 6, 123-130.

[3] Haugland, R. P. (1992-1994) In *Handbook of fluorescent probes and research chemicals*, p45, Molecular Probes Ins. U.S.A.

[4] Scudiero, D. A., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofzinger, T. H., Currens, M. J., Seniff, D. and Boyd, M. R. (1988) *Cancer Res.*, 48, 4827-4833.

[5] Sato, T., Kojima, K., Ihda, T., Sunamoto, J. and Ottenbrite, R. M. (1986) *J. Bioact. Compt. Polym.*, 1, 448-460.

[6] Berlin, R. D. and Oliver, J. M. (1980) *J. Cell Biol.*, 85, 660-671.

[7] Steinman, R. M., Silver, J. M. and Cohn, Z. A. (1974) *J. Cell Biol.*, 63, 949-969.

[8] Suda, Y., Yamamoto, H., Sumi, M., Oku, N., Ito, F., Yamashita, S., Nadai, T. and Ottenbrite, R. M. (1992) *J. Bioact. Compt. Polym.*, 7, 15-24.

[9] Suda, Y., Kusumoto, S., Oku, N., Yamamoto, H., Sumi, M., Ito, F.

and Ottenbrite, R. M. (1992) *J. Bioact. Compt. Polym.*, 7, 275-287.

[10] Thomas, J. L., You, H. and Tirrell, D. A. (1995) *J. Am. Chem. Soc.*, 117, 2949-2950.

[11] Ottenbrite, R. M., Kuus, K. and Kaplan, A. M. (1983) In *Polymers in Medicine*, 3-22, Plenum Pub. co., New York.

[12] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (1994) In *Molecular biology of the Cell*, 483-484, Garland Publishing, Inc., New York.

ABSTRACT

Chapter 1

A capillary affinity gel electrophoresis (CAGE) system was constructed for both separation of oligonucleotides and evaluation of the interaction between oligonucleotides and an immobilized affinity ligand. Poly(9-vinyladenine) (PVAd) was physically entrapped in a gel matrix filling a capillary by *in situ* polymerization of an acrylamide monomer solution containing PVAd. The immobilized PVAd caused retardation of the electrophoretic migration which was specific to oligothymidylic acids (oligo(dT)s). This suggests a base specific interaction between PVAd and oligo(dT)s in the electrophoresis. The migration time of oligo(dT)s was influenced by several parameters; increase of the molecular weight of PVAd or the PVAd concentration delayed the migration, increase of the urea concentration and the capillary temperature decreased the migration time. These observations suggest that the interaction between PVAd and oligo(dT)s should be based on hydrogen bonding. Also through these parameters, separation of oligonucleotides in this CAGE system is highly optimizable. A base-line resolution of 15-mer oligo(dT) from oligo(deoxyadenylic acid)s of similar molecular weights was achieved. The present CAGE system inherits as its advantage both high resolution from CGE and affinity interaction from AGE.

Chapter 2

Using the capillary affinity gel electrophoresis system constructed in the previous chapter, the interaction between an immobilized ligand poly(9-vinyladenine) (PVAd) and oligodeoxynucleotides was investigated. Apparent association constants for the complexation of PVAd and deoxynucleotide hexamers of defined base sequences were obtained. The results suggest that the interaction should occur between the adenine moieties of PVAd and thymidylic acid units of the hexamers and that other bases in the hexamers weaken the interaction as mismatching bases. Two rules governed the strength of the interaction; (i) introduction of a mismatching base in place of T in the sequence of a hexamer decreases the interaction of the hexamer with PVAd, and (ii) a shift of a mismatching in a continuous T sequence toward the center of the T sequence further decreases the interaction. This CAGE system successfully distinguished partial 20-base sequences of a native DNA different by only one base from each other. This demonstrates the validity of the CAGE system for oligonucleotide separation. The above rules were found to be even applicable to the interaction of PVAd with these longer oligodeoxynucleotides which have totally heterogeneous base composition.

Chapter 3

A synthetic polyanionic polymer, poly(maleic acid-alt-7,12-dioxaspiro-[5.6]dodec-9-ene) (MA-CDA), was found to enhance the growth of L929

and STO fibroblasts. After three days, at the optimum polymer concentration, the cell numbers increased 3.7-fold for STO cells and 1.9-fold for the L929 cells compared with the cells cultured in a comparable condition without MA-CDA. The cell growth promotion by MA-CDA required no other external growth factor present in a medium. In addition MA-CDA did not induce the autocrine secretion of growth factors. These results indicate that MA-CDA has a direct growth promotion effect on these fibroblasts. This growth factor-like activity of MA-CDA is novel and unique among known synthetic polymers. Another polymer structurally related to MA-CDA, poly(maleic acid-alt-styrene), also showed the growth promotion activity similar to that of MA-CDA.

Chapter 4

The interactions of cells with poly(maleic acid-alt-7,12-dioxaspiro-[5.6]dodec-9-ene) (MA-CDA) and other polyanionic polymers, which have the growth promotion activity to fibroblasts, were investigated. The polyanionic polymers which show the growth promotion activity have an ability for strong interaction with lipid membrane. The interaction of these polymers different in their structures or molecular weight with lipid membrane was evaluated from perturbation of liposomal membrane by the polymers and showed a good correlation with the growth promotion activities of the polymers previously observed. The result also suggests that hydrophobicity of the

polymers should control the interaction.

Furthermore, uptake of fluorescence labeled MA-CDA (fluorescein MA-CDA) by fibroblasts showed the typical behaviors of endocytic internalization such as suppression by low temperature or by a metabolic inhibitor. Fluorescence miscroscopic observation also supported the endocytosis of fluorescein MA-CDA by L929 cells. In addition suppression of the uptake by unlabeled MA-CDA and the saturation of the uptake at higher MA-CDA concentration (500 µg/ml) suggest binding of MA-CDA to cell plasma membrane as the essential step prior to the internalization.

Acknowledgment

guidance and encouragement through the course of this study.

The author would like to express his deepest gratitude to Associate Professor Yoshinobu Baba and Professor Mitsutomo Tsuhako, Department of Chemistry, Kobe Pharmaceutical University, for their valuable discussion and critical judgment during the course of this study.

The author wish to express his thanks to Associate Professor Kazunari Akiyoshi, Department of Synthetic Chemistry and Biological Chemistry, Faculty of Engineering, Kyoto University, for his intimate discussion on this study.

The author also express his acknowledgments to Associate Professor Akio Kishida, Department of Applied Chemistry, Faculty of Engineering, Kagoshima University for helpful discussion.

The author would like to express his sincere gratitude to Professor Raphael M. Ottenbrite, Department of Chemistry, Virginia Commonwealth University, for his intimate discussion and significant suggestion.

The author also thanks to all members of Professor Sunamoto Laboratory and Professor Akashi Laboratory for their kind help, generosity, disinterested friendship.

May, 1996

Tomohiro Sawa

LIST OF PUBLICATIONS

Chapter 1
"Specific Base recognition of oligodeoxynucleotides by capillary affinity gel electrophoresis using polyacrylamide-poly(9-vinyladenine) conjugated gel".
Y. Baba, M. Tsuhako, T. Sawa, M. Akashi, & E. Yashima.
Anal. Chem. (1992) 64, 1920-1925.

"Temperature-programmed capillary affinity gel electrophoresis for the sensitive base-specific separation of oligodeoxynucleotides".
Y. Baba, M. Tsuhako, T. Sawa, & M. Akashi.
J. Chromatogr. (1993) 632, 137-142.

"Effect of urea concentration on the base-specific separation of oligodeoxynucleotides in capillary affinity gel electrophoresis".
Y. Baba, M. Tsuhako, T. Sawa, & M. Akashi.
J. Chromatogr.(1993) 652, 93-99.

Chapter 2
"Specific separation of oligodeoxynucleotides by capillary affinity gel electrophoresis (CAGE) using poly(9-vinyladenine)-polyacrylamide conjugated gel".
M. Akashi, T. Sawa, Y. Baba, & M. Tsuhako.
J. High Resol. Chromatogr.(1992) 15, 625-626.

"Evaluation of the selective binding ability of oligodeoxynucleotides to poly(9-vinyladenine) using capillary affinity gel electrophoresis".
Y. Baba, H. Inoue, M. Tsuhako, T. Sawa, A. Kishida, and M. Akashi.
Anal. Sci.(1994) 10, 967-969.

Chapter 3

"Enhanced growth of cultured fibroblasts by a synthetic polyanion polymer".
T. Sawa, Y. Okumura, J.-L. Ding, R. M. Ottenbrite, & J. Sunamoto.
J. Bioact. Compt. Polym.(1996) 11, 17-27.

Chapter 4

"Binding and endocytosis as the primary interaction between polyanionic polymers and fibroblasts".
T. Sawa, Y. Okumura, J.-L. Ding, R. M. Ottenbrite, & J. Sunamoto.
J. Bioact. Compt. Polym. to be submitted.

LIST OF OTHER PUBLICATIONS

"High-performance affinity chromatography of oligonucleotides on nucleic acid analogue immobilized silica gel columns".
E. Yashima, T. Shiiba, T. Sawa, N. Miyauchi, & M. Akashi.
J. Chromatography. (1992) 603, 111-119.

"A synthetic polyanion polymer as a growth promoter for L929 mouse fibroblast".
T. Sawa, Y. Okumura, J.-L. Ding, R. M. Ottenbrite, & J. Sunamoto.
In *Advanced Biomaterials in Biomedical Engineering and Drug Delivery Systems*, N. Ogata, S. W. Kim, J. Feijen, and T. Okano (Eds) (1996) P285-286, Springer-Verlag Tokyo.