# Synthesis of DNA Conjugate by Mechanochemical Solid-State Polymerization and Its Affinity Separation of Oligonucleotides Having Single-Base Difference by Capillary Electrophoresis

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**In this communication, we discuss the characterization of DNA conjugate synthesized by mechanochemical polymerization, Con-M, on the separation of model oligo-DNA and its single nucleotide polymorphisms (SNPs) by affinity capillary electrophoresis, compared with that prepared by radical-initiated solution polymerization, Con-RL. The average molecular weight of Con-M was similar to that of Con-RL, although the molecular weight distribution of Con-M was narrower than that of Con-RL. Capillary electrophoresis of oligo-DNA was performed using the capillary filled with DNA conjugate. The resolution of the capillary filled with Con-M was apparently higher than with Con-RL. It is considered that higher resolution using the capillary filled with Con-M could be ascribed not only to the narrow molecular weight distribution but also to the difference of copolymer structure.**

**Key words** DNA conjugate; mechanochemical polymerization; affinity capillary electrophoresis; single nucleotide polymorphism; copolymer structure

Information about DNA sequence variation will have a wide range of applications for analyzing disease and developing diagnostic, therapeutic and preventative strategies. DNA polymorphisms such as single nucleotide polymorphism (SNP) will be useful in helping researchers determine and understand why individuals differ in their abilities to absorb or clear certain drugs, as well as why an individual may experience an adverse side effect to a particular drug. Therefore, the recent discovery of SNPs promises to revolutionize not only the process of disease detection but also the practice of preventative and curative medicine. Maeda *et al.* have developed an affinity capillary electrophoresis for gene mutation assay using oligonucleotide-pendant polyacrylamide (DNA conjugate), which is synthesized by radical-initiated solution polymerization, as a pseudo-immobilized affinity ligand. This system can separate oligonucleotide (oligo-DNA) having an oncogene sequence and its SNP.[1—5)]

We have reported the syntheses and the nature of drug release of polymeric prodrugs prepared by mechanochemical solid-state polymerization,[6—14)] which is carried out by vibratory ball-milling of solid monomer in a metallic vessel under an aerobic condition. Several important conclusions were reached from a series of such studies. The monomers prepared based on the criteria derived from quantum chemical considerations underwent facile mechanochemical solid-state polymerizations to give the corresponding polymeric prodrugs essentially quantitatively. Thus, this method eliminates the need for any work-up of the reaction mixture.[6,9)] One of the most striking properties observed in polymers obtained is that the resulting polymeric prodrugs are of very low heterogeneity (narrow molecular weight distribution) represented by $M_w/M_n$, ($M_w$, weight average molecular weight; $M_n$, number average molecular weight), which is of great value in pharmaceuticals for highly functionalized polymeric prodrugs. The mechanochemical solid-state copolymerization provides multi-block copolymer, since the propagation reaction proceeds in a crystal of monomer (Fig. 1). This is apparently contrastive to the fact that the random copolymer is usually produced by the radical-initiated solution polymerization. It is also shown that the mechanochemical solid-state copolymerization is an ideal copolymerization under the appropriate operational condition, providing a copolymer that is homogeneous in composition. Therefore, the present reactions seem to be applicable to a wide variety of vinyl monomers of an important class of bioactive compounds with different physicochemical properties and provide a novel and simple methodology for synthesis of functionalized polymer through a totally dry process.[11)]

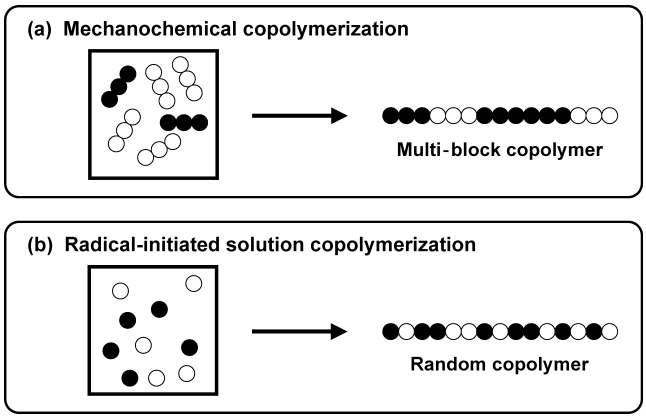


Fig. 1. Conceptional Illustration of Polymer Structures Prepared by (a) Mechanochemical Copolymerization and (b) Radical-Initiated Solution Copolymerization

When DNA conjugate is used as an affinity ligand of capillary electrophoresis for gene mutation assay, it is considered that the average molecular weight and molecular weight distribution of DNA conjugate could be one of the factors affecting the separation of sequence isomers of oligo-DNA which have the same chain length but have different base sequences. As described above, the polymer synthesized by mechanochemical solid-state polymerization possesses various unique properties such as narrow molecular weight distribution, so that this method could be useful for the fabrication of DNA conjugate with higher separation. In this communication, we describe the characterization of DNA conjugate synthesized by mechanochemical polymerization on the separation of model oligo-DNA and its SNPs by affinity capillary electrophoresis, compared with that prepared by conventional radical-initiated solution polymerization. The synthesis of DNA conjugate is outlined in Chart 1.

First the copolymer of acrylamide (AAm) and methacryloyloxy succinimide (MAS) was synthesized by mechanochemical solid-state copolymerization or radical-initiated solution copolymerization. The mechanochemical solid-

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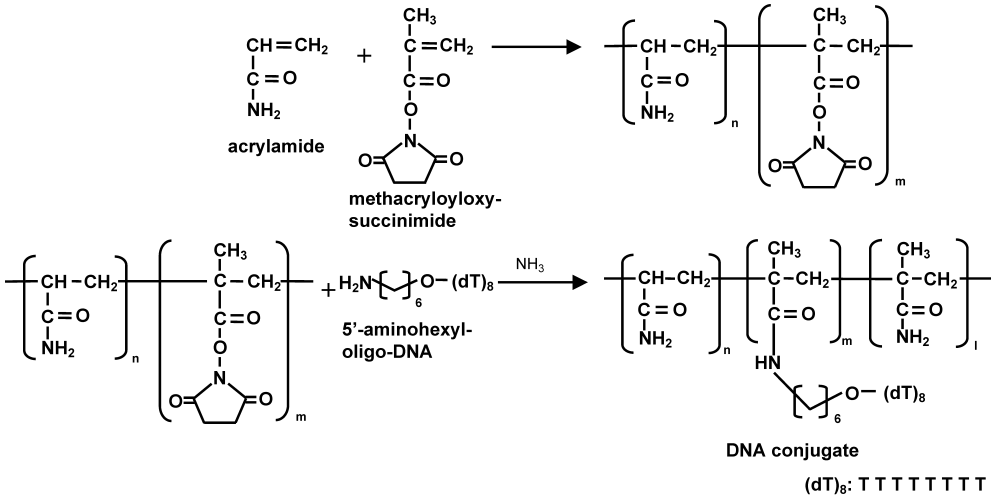


Chart 1

state copolymerization of AAm and MAS was carried out as follows: The mixture of AAm (88.1 mg) and MAS (11.9 mg), which contained 5 mol% MAS, was mechanically fractured by vibratory ball-milling (stainless steel ball (6.0 mm$\phi$, 890 mg) in a stainless steel twin-shell blender (10 mm$\phi$, 22.5 mm long) (Mixer Mill MM2000, Retsch GmbH & Co. KG)) at room temperature for 2 h at 30 Hz under an aerobic condition. The disappearance of vinyl proton and the appearance of the corresponding alkyl proton were observed in the $^1$H-NMR spectrum of the resulting fractured powder, so that it was confirmed that this reaction proceeded to completion. Therefore, the copolymer synthesized by mechanochemical copolymerization, Poly-M, contained 5 mol% MAS. The average molecular weight and molecular weight distribution of Poly-M were measured by a gel permeation chromatograph (GPC, Shimadzu LC-6A) equipped with a refractive index detector (Shimadzu, RID-6A), gel column (Shodex, KD-800M and KD-80M), and a data analyzer (Shimadzu, Chromatopac C-R4A), under the following conditions: elution solvent, dimethylformamide (DMF) containing 10 mmol LiBr; flow rate, 0.7 ml/min; column temperature, 40 °C. Calibration was carried out with a standard specimen of poly(ethylene oxide). The number average molecular weight ($M_n$) and heterogeneity ($M_w/M_n$) of Poly-M were 30000 and 1.21, respectively. Thus, Poly-M possessed the narrow molecular weight distribution. The radical-initiated solution polymerization was carried out as follows: AAm (881 mg), MAS (119 mg) and $\alpha,\alpha'$-azobisisobutyronitrile (2 mg) in DMF (3 ml) was warmed at 60 °C in a sealed glass-made tube under nitrogen for 90 min. The content was poured into a large amount of acetone (90 ml). The precipitated polymer was collected and dried *in vacuo* to yield 70 mg (7%). The elemental analysis of this copolymer, Poly-RH, revealed that Poly-RH contained 5 mol% MAS. The average molecular weight and heterogeneity of Poly-RH were also measured by GPC, so that Poly-RH were $M_n=70000$ and $M_w/M_n=2.00$. To obtain the copolymer of lower molecular weight, Poly-RH (100 mg) was fractured for 2 h at 30 Hz with a stainless steel twin-shell blender. The fractured polymer, Poly-RL, was $M_n=32000$ and $M_w/M_n=1.63$.

To a solution of Poly-M (10 mg) in pH 7.4 Tris–borate (TB) buffer (1 ml) was added a solution of 5′-aminohexyl-$(dT)_8$ (20 $\mu$l, 1.88 mM TB buffer solution). The mixture was stirred at room temperature overnight. One drop of 25% ammonia solution was added to the mixture. The DNA conjugate solution, Con-M, was prepared. By the same procedure, the DNA conjugate solution, Con-RL, was prepared from Poly-RL.

Free zone capillary electrophoresis of the resulting DNA conjugate solution was carried out at 260 nm. This method can separate and determine a free 5′-aminohexyl-$(dT)_8$ and DNA conjugate, so that the yield of reacted 5′-aminohexyl-$(dT)_8$ can be estimated. Ninety percent of 5′-aminohexyl-$(dT)_8$ in the reaction mixture reacted with Poly-M or Poly-RL under this experimental condition, indicating that the amount of $(dT)_8$ incorporated to both DNA conjugates, Con-M and Con-RL, was 0.02 mol%. Thus, the average molecular weight and the DNA content of Con-M were similar to those of Con-RL, although the molecular weight distribution of Con-M was slightly narrow.

The capillary was precoated with linear polyacrylamide according to the literature.[15] The resulting capillary (50 cm) was filled with Con-M or Con-RL in 30 cm length from the cathodic end and the rest of the capillary was filled with 1% polyacrylamide (Kishida Chemical Co., Ltd, average molecular weight 9000000—10000000). Then the capillary was charged with $-15$ kV to remove the unreacted 5′-aminohexyl-$(dT)_8$. Electrophoresis of sample oligonucleotides was performed using the capillary in 10 mM Tris–borate buffer (pH 7.4) containing 75 $\mu$M $MgCl_2$. Sample solution was introduced into the capillary at the cathodic side by positive pressure (0.1 kgf/cm$^2$) for 3 s and was charged with $-15$ kV constant voltage.

Figure 2 shows electropherograms on the separation of two oligonucleotides. Three kinds of oligonucleotides, $(dA)_8$, $(dA)_4(dT)(dA)_3$ and $(dA)_6(dT)(dA)$, were used in this experiment. $(dA)_8$ is the perfect match sequence to the affinity ligand $(dT)_8$, while $(dA)_4(dT)(dA)_3$ and $(dA)_6(dT)(dA)$ have one mismatched base but the mismatch position is different from one another. It is known that $Mg^{2+}$ enhances the ligand-analyte interaction due to stabilizing double-stranded form of DNA,[1—3] so that $Mg^{2+}$ was added to working solution.

By using the capillary filled with Con-M, $(dA)_8$ was successfully separated from its single base mutant, $(dA)_4(dT)$

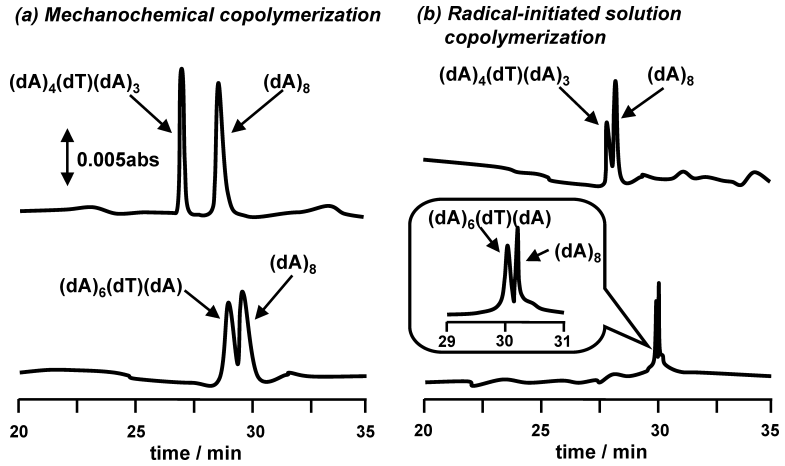


Fig. 2. Separation of Model Oligo-DNA (5′-AAAAAAAA-3′) and Its SNPs (5′-AAAATAAA-3′ or 5′-AAAAAATA-3′) Using Capillary Filled with DNA Conjugate Synthesized by (a) Mechanochemical Copolymerization and (b) Radical-Initiated Solution Copolymerization

Working solution, 10 mM Tris–borate (pH 7.4) + 75 $\mu$M $MgCl_2$.

$(dA)_3$ or $(dA)_6(dT)(dA)$, as shown in Fig. 2a. Although electropherograms using the capillary filled with Con-RL also showed two peaks assigned to $(dA)_8$ and its single base mutant (Fig. 2b), the resolution of this capillary was apparently lower than that of the capillary filled with Con-M. It is known that the degree of peak retardation has a good correlation with the stability of the duplex between each analyte and the pendant affinity ligand.[1—5] Thus, $(dA)_6(dT)(dA)$, which has a longer consecutive sequence of dA, showed larger retardation than $(dA)_4(dT)(dA)_3$ in both cases. As described above, radical-initiated solution copolymerization generally gives a random copolymer, and mechanochemical solid-state copolymerization produces a multi-block copolymer. Thus, it is considered that higher resolution of the electrophoresis using the capillary filled with Con-M could be ascribed not only to the narrow molecular weight distribution but also to the difference of copolymer structure.

We are now actively elaborating this initial study. It is hoped that DNA conjugate possessing higher resolution will be designed in the course of attempt now in progress.

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