Flowable Networks as Equilibrium DNA Sequencing Media in Capillary Columns

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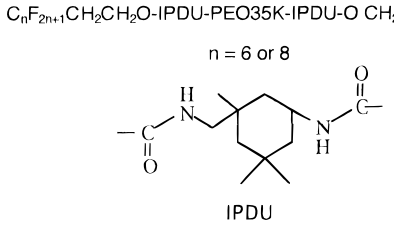
Two strategies are currently used for the sieving component of DNA sequencing separation media. In slab format, gel electrophoresis on chemically cross-linked polyacrylamide (polyacrylamide gel electrophoresis, PAGE) provides high performance and substantial dynamic range.[1,2] PAGE gels in capillary columns showed high resolution, but have the shortcoming that the columns cannot be refilled. For capillary electrophoresis (CE), solutions of un-cross-linked entangled linear water-soluble polymers are gaining favor.[3] Both of these techniques rely on the intrinsic "mesh size" (ξ_{eff}) of the sieving polymer for DNA separation.[4] This size is a function of the polymer chain concentration (c_{pol}) and stiffness.[5] The effective mesh size of a chemically cross-linked polymer depends on both the amount of cross-linker added to the reaction mixture and the detailed kinetics of the polymerization reaction.[6] For a linear, entangled polymer system, the largest change of mesh size occurs from the overlap concentration (c^*) to about 5 times c^*.[7] The high-resolution performance required for sequencing applications normally involves $c_{pol} > 10c^*$, a regime where changes in ξ_{eff} with concentration are quite small. It is more effective to increase chain length, thus lowering c^*,[5] but large molecular weight changes are required.[8]

Here we describe an alternative strategy that involves linear water-soluble polymers with strongly associating fluorocarbon end groups. These systems form flowable networks of self-assembled gels, in which the mesh size appears to be controlled by the chain length between the associating groups, and to a lesser extent, by the polymer concentration. These micelle-based systems are effective as CE separation media for single-stranded DNA under denaturing conditions. Because of the self-assembling nature of the gel, one can achieve large mesh sizes with relatively low molecular weight polymers. For this study we chose PEG35 000 end capped with either $C_6F_{13}C_2H_4O-$ groups (C_6F-35K) or $C_8F_{17}C_2H_4O-$ groups (C_8F-35K), prepared through reaction of the corresponding alcohol first with isophorone diisocyanate, and then with PEG. The structure of these polymers is



Experimental Section

Materials and Rheology. $C_6F_{13}C_2H_4OH$ and $C_8F_{17}C_2H_4$-OH (PCR Inc.) were reacted with a large excess of isophorone diisocyanate (Aldrich), and the excess was then removed under vacuum. This monoisocyanate in excess was reacted with PEG35 000 (Fluka) in dry diglyme at reflux in the presence of dibutyltin laurate until the reaction went to completion. Full details of the synthesis and characterization of the polymers will be reported elsewhere.[9] By a combination of ^{1}H and ^{19}F NMR, the polymer was found to have 2.0 ± 0.05 end groups/chain. Response to steady and oscillatory shear were measured on a Rheometrics RAA 2 analyzer with a cone and plate geometry (50 mm, 0.04 rad cone angle).

DNA Sequencing. The DNA sequencing sample was generated from M13mp18 single-stranded template primed with −21M13 using dye-labeled dideoxy terminators labeled with R110, R6G, TAMRA, and ROX on G, A, T, and C, respectively. All reagents were obtained from a Taq DyeDeoxy Terminator Cycle Sequencing Kit (part no. 901497) purchased from Applied Biosystems-Perkin Elmer, and the protocol followed the user bulletin associated with this kit. The sequencing matrix was 1:1 C_6F-35K:C_8F-35K (6 wt % in 40% urea and 100 mM sodium 3-tris(hydroxymethyl)methylamino-1-propane sulfonate (TAPS) at pH 8.0). The internally coated (DB-210) capillary (47 cm, 75 μm i.d.) was purchased from J and W Scientific, Folsom, CA. Fluorescently labeled DNA sequencing sample was suspended in formamide, electrokinetically injected, and electrophoresed at 200 V/cm. Signal was detected 36 cm from the cathode end with laser-induced fluorescence using the 488 and 514 nm lines of a 10 mW argon ion laser and a CCD spectrometer for fluorescent light collection; raw data accumulated as CCD signal was multicomponent-analyzed and treated with Applied Biosystems-Perkin Elmer version 2.0 sequencing analysis software to yield the relative concentration of each dye-labeled extension product.

Results and Discussion

Flowable Gels. Water-soluble polymers bearing hydrophobic end groups are employed in the paint industry as rheology modifiers. One of their key characteristic features is that they show classical Newtonian flow at low shear rates and undergo pronounced shear thinning once the shear rate exceeds a critical value. Recent studies on model polymers demonstrate that these types of polymers form rapidly equilibrating self-assembling gels.[10−14] For PEG polymers with $C_{16}H_{33}O-$

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(2) (a) Resolution of polynucleotide fragments from 30 to 800 bases long, differing in length by a single base, is attainable in a single experiment (b) Ansorge, W.; Barker, R., *J. Biochem. and Biophys. Methods* **1984**, *9*, 33. (c) Lang, B. F.; Burger, G. *Anal. Biochem.* **1990**, *188*, 176. (d) Nishikawa, T.; Kambara, H. *Electrophoresis* **1991**, *12*, 623. (e) Grossman, P.; Menchen, S.; Hershey, D. *Gen. Anal.: Tech. Appl.* **1992**, *9*, 9−16.
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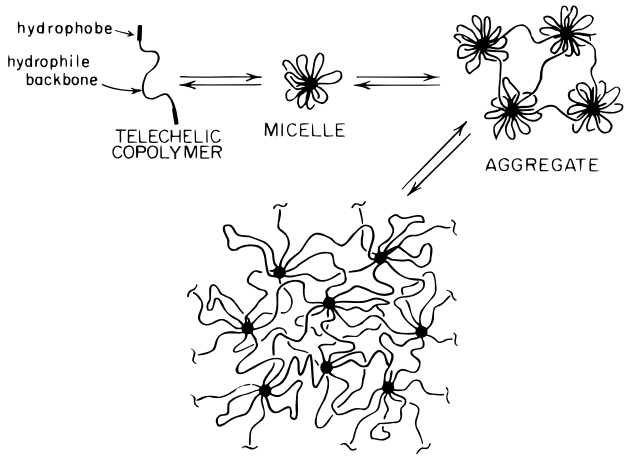

Figure 1. Structures formed from water-soluble polymers with hydrophobic end groups. Above the cmc, polymer associates into rosette-like micelles. At higher concentrations, higher aggregates form through bridging. As aggregates become larger and more concentrated, a continuous network is generated.

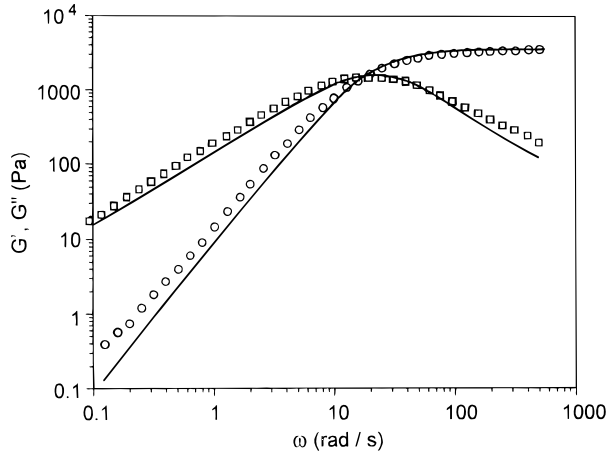

Figure 2. Plot of G' and G'' for a 5.0 wt % solution of C_8F-35K in water at 25 °C. The lines represent the best fit to a single-element Maxwell model, with τ and $G_n°$ as the fitting parameters.

end groups attached to the polymer by reaction with isophorone diisocyanate, association begins at a very low concentration to form spherical flowerlike micelles comprised of looped chains. At higher concentrations (c_{pol} = 0.5−1 wt %) they rearrange to form bridged aggregates (cf. Figure 1), which at c_{pol} > 2 wt % fill space to form a cross-linked superstructure with the viscoelastic properties of a gel and with a mesh size dependent on the composition of the hydrophilic polymer separating the hydrophobic chain ends.

We find similar rheological behavior for both C_6F-35K and C_8F-35K: The zero shear viscosity increases by several orders of magnitude as the concentration is increased, with a concentration-dependent onset of shear thinning. An indication of structure buildup in the system is inferred from the strong dependence of the zero-shear viscosity (η_0) on concentration: $\eta_0 \sim c_{pol}^x$, with x = 2.4 for C_6F-35K and x = 4.1 for C_8F-35K. Nevertheless, these solutions exhibit a very simple viscoelastic behavior, which can be described in terms of a single-element Maxwell model, characterized by a plateau modulus ($G_n°$) and a single relaxation time (τ). One set of data showing the fit of the elastic (G') and the loss (G'') moduli to this model is shown in Figure 2. Note that the zero-shear viscosity $\eta_0 = G_n°\tau$.[10] At all concentrations, the $G_n°$ values of C_6F-35K and C_8F-35K are remarkably similar (2800 vs 2900 Pa, respectively, at 5 wt %), and the lower viscosity of the former is due primarily to its faster relaxation time (1.4 vs 66 ms at 5 wt %). This time corresponds to exit of the hydrophobic group from the micelle and is the slowest step in the rearrangement of the network, an idea developed by Annable[10] in terms of the transient network model. Both $G_n°$ and τ increase with concentration, but during the fill time of the capillary (minutes), the system comes rapidly to equilibrium. This is in strong contrast to PAGE gels, where the chemical cross-links lock in the gel structure formed under kinetic control during the polymerization reaction.

Several observations are critical to understanding the usefulness of the system for refilling capillaries for DNA sequencing. First we find that the refill time or, alternatively, the pressure drop needed for refill, is increased markedly when the capillary is coated with a thin layer of fluorocarbon modified polysiloxane film (J and W, D-210), indicating strong adhesion of the end groups to the capillary wall. Second, we find that in the shear thinning region, the shear stress becomes independent of the shear rate. The onset of shear thinning is accompanied an increase of the normal force, which drops at higher shear rates. These results suggest that at high shear rates, there is a breakdown of the gel structure in a layer near the capillary wall to create a lubricating layer, resulting in a plug flow of the contents from the tube. Slip flow for polymer solutions is rare but not unprecedented.[15] Here this behavior is very useful from the point of view of rapidly refilling the capillary and expelling from the column all material from a previous sequencing experiment.

DNA Sequencing. Both C6F-35K and C8F-35K give good sequencing results, with a limit of resolution of ca. 350 bases.[16] Curiously, a 1:1 mixture of these polymers, a fluid easily pumpable through the 75 μm i.d. capillary at moderate pressures,[17] gives enhanced resolution (440 bases). Polymer concentration has a rather small effect on resolution, and operates on the peak-to-peak separation in much the same way one finds for PAGE gels.[18]

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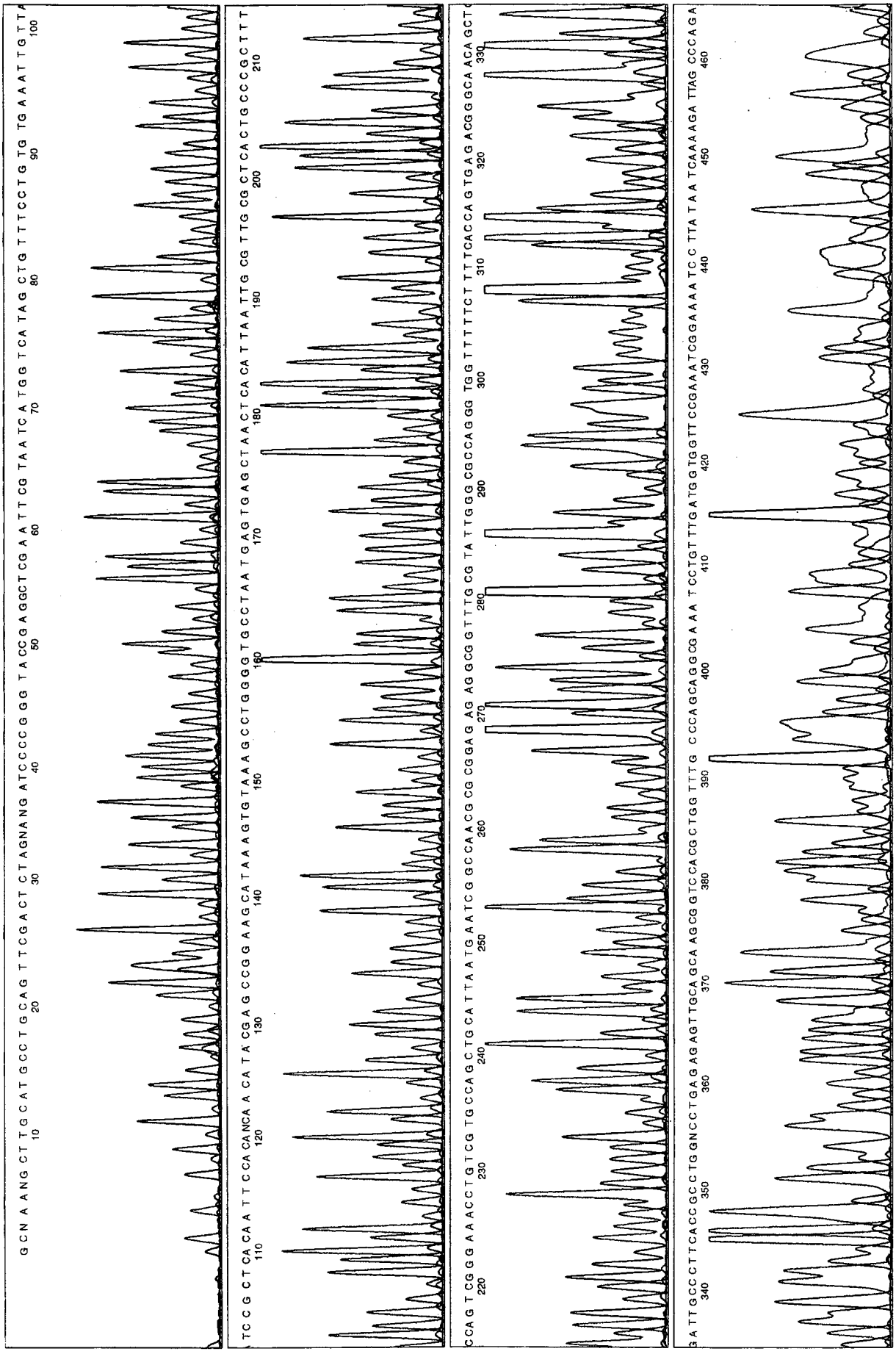

Figure 3. Four-color DNA sequencing in micellar networks. The DNA sequencing sample was generated from M13mp18 single-stranded template primed with -21M13 using dye-labeled dideoxy terminators labeled with R110, R6G, TAMRA, and ROX on G, A, T, and C, respectively. For full details, see ref 9a. The sequencing matrix was a 1:1 mixture of C_6F-35K and C_8F-35K (6 wt %) in 40% aqueous urea and 100 mM sodium 3-tris(hydroxymethyl)methylamino-1-propane sulfonate (TAPS) at pH 8.0. Raw data accumulated as fluorescent signal was multicomponent analyzed by Applied Biosystems-Perkin Elmer version 2.0 sequencing analysis software to yield the relative concentration of each dye-labeled extension product.

An example of four-color automated sequencing is shown in Figure 3. After each run, the fluid is replenished, and a new sample is electrokinetically injected onto the column. There is no carryover of residue from one filling to the next, a likely consequence of the unusual shear-thinning characteristics of the self-assembling fluid. This type of behavior is essential for automated sequencing in capillaries.

In summary, PEG polymers with fluorocarbon end groups serve as effective sieving media for electrophoretic sequencing of DNA. The end groups self-assemble reversibly into micelle-like structures, generating equilibrium structures that ensure run-to-run reproducibility. On the basis of our results, we anticipate improved sequencing performance for PEG chain lengths beyond molecular weight 35 000, which should increase the mesh size. We also suggest that modifying other water soluble polymers such as polyacrylamide with strongly associating end groups will also form flowable transient networks.

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