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Review

# Macroporous gels: facts and misfacts

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**Abstract**

Some extraordinary events have occurred in the last two years which might revolutionize the field of polyacrylamide gel electrophoresis. While it was widely recognized that such matrices could normally be cast with a small pore size distribution, typically of the order of a few nanometres diameter (for protein sieving), recent developments suggest that "macroporous" gels could also be produced in the domain of polyacrylamides. If constraints on chain motion are imposed during gel polymerization, large-pore structures can be grown. Such constraints can originate either from low temperatures or from the presence of preformed polymers in the gelling solution; in both instances, the growing chains are forced to "laterally aggregate" via inter-chain hydrogen bond formation. On consumption of pendant double bonds, such bundles are frozen in the three-dimensional space by permanent cross-links. As an additional development, a novel photopolymerization system is described, consisting of a cationic dye (methylene blue) and a redox couple (sodium toluene sulphinate, a reductant, and diphenyliodonium chloride, a mild oxidant). Methylene blue catalysis is characterized by a unique efficiency, ensuring >96% conversion of monomers even in aqueous–organic solvents and in presence of surfactants, which normally quench or completely inhibit the peroxodisulphate-driven reaction. In addition, methylene blue-sustained photopolymerization can be operated in the entire pH range 3–10, where most other systems fail. Perhaps the most striking novelty in the field is the appearance of a novel monomer (N-acryloylaminoethoxyethanol, AAEE) coupling a high hydrophilicity with a unique resistance to alkaline hydrolysis. Given the fact that a poly(AAEE) matrix is 500 times more stable than a poly(acrylamide) gel, while being twice as hydrophilic, it is expected that this novel chemistry will have no difficulties in replacing the old electrophoretic anticonvective media. The review ends with a glimpse at novel sieving media in capillary zone electrophoresis: polymer networks. Such media, by providing an almost infinite range of pore sizes, owing to the absence of a rigid support, allow sieving mechanisms to be operative over a wide interval of particle sizes, even up to genomic DNA. Viscous solutions of polymer networks, made with the novel poly(AAEE) chemistry, allow the repeated use of the same separation column, well above 50 injections. Silica-bound poly(AAEE) chains provide effective quenching of electrosmosis and >200 analyses by isoelectric focusing.

## Contents



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## 1. Preface

First get down upon your knees
fiddle with your rosaries
bow your head with great respect
and genuflect, genuflect, genuflect

[From Tom Lehrer: *The Vatican Rag* (Reprise, RS-6179)]

Thus spoke my friend Tom (in fact sung, in fact played at the piano in a peppery rag tune). When I was a young post-doc. in Cambridge (MA, USA) my mathematics teacher Tom Lehrer (at Harvard) used to entertain his students as much in mathematics as in music. In those years, in fact, anybody believing he had or could produce macroporous polyacrylamide gels needed a lot of religious belief and a lot of fiddling with rosaries to produce such a miracle. In 1965 (when the album *That Was The Year That Was* by Lehrer was released) it was barely understood that polyacrylamides, if at all, had small pore sizes, of the order of a few nanometres, in fact well suited for sieving such (relatively) small particles as proteins [1–5]. The situation has changed a lot nowadays.

## 2. Introduction

In the 1960s, as stated above, it was understood that, because protein particles were effectively sieved in standard polyacrylamide gels (typical formulations of 5%T, 5%C), such gels should have small pore diameter, on average *ca.* 5 nm [1–5]. However, there has not been much of a consensus on what kind of equations would best describe the average pore radii $\langle a \rangle$ as a function of the polyacrylamide concentration (%T). Some of the reported relationships are

$$\langle a \rangle = Kd/(\%\mathrm{T})^{0.5} \quad \text{(ref. 6)}$$

$$\langle a \rangle = Kd/(\%\mathrm{T}) + K' \quad \text{(ref. 5)}$$

$$\langle a \rangle = 1/(\%\mathrm{T})^{0.33} \quad \text{(ref. 7)}$$

In contrast, it was immediately apparent that agarose matrices had in fact a much larger pore size. How much larger, though, was not known until we started "titrating" the pore size by driving sulphated polystyrene particles through the gel [8]. As shown in Fig. 1, the largest pore diameter (as measured by assessing the size of

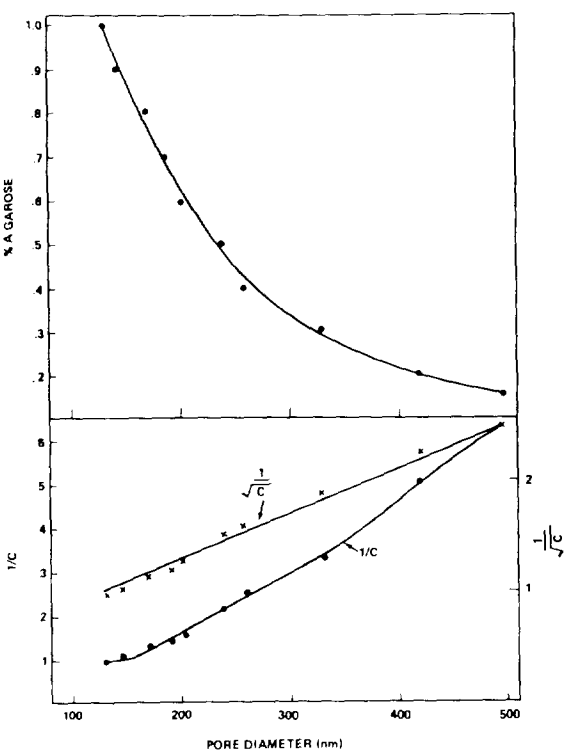


Fig. 1. Plot of limiting pore size *vs.* percentage of agarose. The experimental points in the upper part are the pore diameters obtained by driving electrophoretically latex particles in gels of 0.16 to 1.0% agarose and extrapolating a particle diameter corresponding to "zero net migration". In the lower part, the experimental points have been replotted according to the scaling law $C^{-0.5}$ (right-hand scale) or according to $C^{-1}$ (left-hand scale). Note that the upper curve only fits the scaling law $C^{-0.7}$. From ref. 8.

the latex which would just be arrested at the deposition site) one could obtain, in the more dilute agarose gel (which in that study was 0.16%), was 400 nm. This meant an increment of about two orders of magnitude compared with standard polyacrylamides. We also gave an equation linking the average pore diameter $\langle d \rangle$ (in nm) to the concentration ($C$, in %) of the agarose powder in the gel:

$$\langle d \rangle = 140.7C^{-0.7}$$

Later, a number of equations (reviewed by Noolandi [9]) were given correlating the pore radii $\langle a \rangle$ to $C$:

$$\langle a \rangle = 117.4/C^{1.71}$$

$$\langle a \rangle = 122C^{0.8}$$

$$\langle a \rangle = 157/C^{1.04}$$

$$\langle a \rangle = 25 + 70/C$$

$$\langle a \rangle = 89/C^{0.65}$$

It is seen that the exponents differ substantially, which would lead to widely divergent pore sizes for the same gel matrix. Unknown to all the above workers, in 1979 De Gennes [10] addressed the same problem and derived a universal exponent linking the pore size to the agarose concentration ($C$):

$$\langle a \rangle = lC^{-0.75}$$

where $l$ is the statistical segment length. This equation was derived for yet another condition, *i.e.*, not just a chemical gel, forming a solid mass, but for a physical gel, *i.e.*, a viscous solution of linear polymers (called a polymer network) above a critical concentration named the "entanglement threshold". This pore size is then referred to as an "average mesh size". At or above this critical threshold, the mesh size is about the same as the radius of gyration ($R_g$) of an individual polymer coil and it scales as $C^{-0.75}$ [11]. It is of interest that a few of the above equations, as listed by Noolandi [9], are in fact centred around this universal exponent. As work progressed on agaroses [12], it become possible to gel such matrices as extremely dilute, almost liquid phases (only 0.03% solids); in such semi-liquid supports, Serwer *et al.* [12] reported penetration even of an *Escherichia coli* cell (a particle $0.5 \times 2$ $\mu$m in size). Today, there is great interest in performing separations in viscous polymer solutions, especially in capillary zone electrophoresis (CZE), where the sieving solution is physically contained and held there by capillary forces inside the separation chamber (tubes of 20–150 $\mu$m bore) [13]. Interestingly, this idea of De Gennes [10], of sieving in viscous polymer solutions, which was experimentally verified during the same period by Bode [14,15], was already ingrained in a concept promulgated by Ogston [16], who stated that, "real cross-linked gels have thermodynamic properties, both in themselves and in their interaction with solute molecules, very similar to those of equivalent solutions of uncrosslinked molecules". Thus, at present, there is a general consensus that there is a dichotomy between the two most popular gel media in electrophoretic separations: agarose gels are used almost exclusively for nucleic acid fractionation [17,18], whereas polyacrylamides have become the matrix of choice for protein analysis [19]. Yet the situation might be more complex than that. Already in 1981, Righetti *et al.* [8] demonstrated the possibility of producing "macroporous" polyacrylamides simply by gelling a low %T matrix, but having a very high %C content (up to 60% C). As shown in Fig. 2, such a matrix could attain a porosity as high as 600 nm average diameter, even higher than the agarose matrices reported up to that time. The concept of producing relatively large pore size polyacrylamides by increasing the %C was already implicit in the classical paper by Davis [20], who suggested, for a large-pore stacking gel, a matrix consisting of 2.5% T cross-linked with 20% bisacrylamide (a fact that has escaped the attention of most users). However, this turned out to be a dead-end; as already recognized by Righetti and co-workers [8,21], such highly cross-linked, highly porous polyacrylamides were in fact useless for all practical purposes, not only because they were opaque, but also because they were too hydrophobic and thus would adsorb proteins and exude water. Even when cross-linking with DHEBA [N,N'-

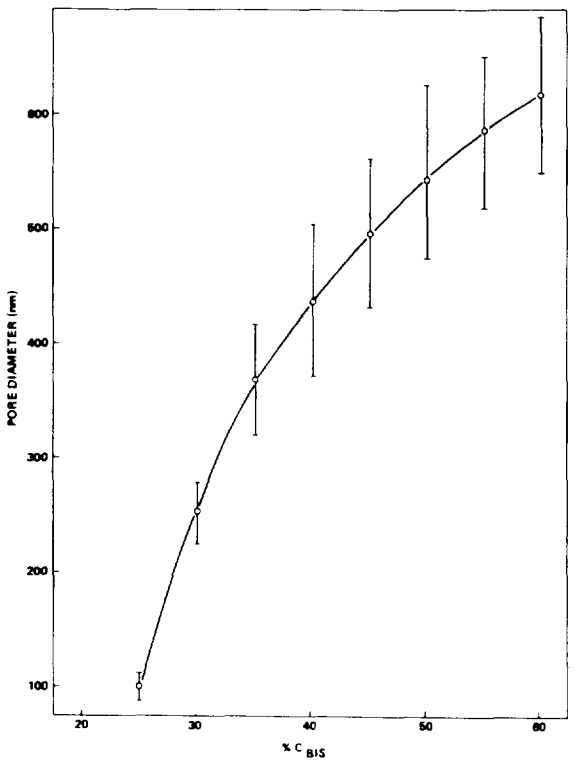


Fig. 2. Plot of pore diameter *vs.* percentage of cross-linker (Bis) in polyacrylamide gels of 3% T. The experimental points are the pore diameters obtained by driving electrophoretically latex particles in gels of 3% T, ranging in amount of cross-linker from 25 to 60% C, and extrapolating a particle diameter corresponding to "zero net migration". The vertical bars represent the standard deviation. From ref. 8.

(1,2-dihydroxyethylene)bisacrylamide], a much more hydrophilic cross-linker, the situation was not ameliorated, suggesting that the solvent loss was due to intrinsic difficulties of water in penetrating the tightly packed bundles of gel fibres, ultimately responsible for such a huge increment in pore size.

## 3. Macroporous polyacrylamides: polymer-induced lateral aggregation

Even though the above process for producing "macroporous" gels had to be abandoned, some interesting observations were useful. First, the Ogston [22] theory predicted that, if the average polyacrylamide fibre were to be reduced in length and thickened in diameter (believed to be of the order of 0.5–1.0 nm), the average pore size would increase progressively. In fact, when Davis [20] proposed 20% C matrices for the sample and stacking gel segments, Fawcett and Morris [23] found that, in such high %C gels, the fibre diameter would increase from 0.5 to 6 nm, as a result of "bundling" of individual fibres into "pillar-like" aggregates. Second, we could link this bundling phenomenon to an increased gel turbidity: polyacrylamide gels, known to be completely transparent in the visible light spectrum, became progressively opaque as the %C was increased from 10% to 60%. We then recently chanced upon a curious phenomenon: when gelling a polyacrylamide in the presence of another polymer [*e.g.*, polyethylene glycol (PEG), polyvinylpyrrolidone, hydroxymethylcellulose], turbid gels were produced. The turbidity was a function of both length and concentration of the polymer in the gelling solution. Fig. 3 shows the extent of this phenomenon in solutions of PEG ranging in relative molecular mass ($M_r$) from 2000 up to 20 000; it is seen that longer polymer chains induce this transition at much lower concentrations (*ca.* 1.2% in $M_r$ 20 000 PEG, *vs.* >10% in $M_r$ 2000 PEG).

By studying this process in depth, we concluded that the preformed polymer present in

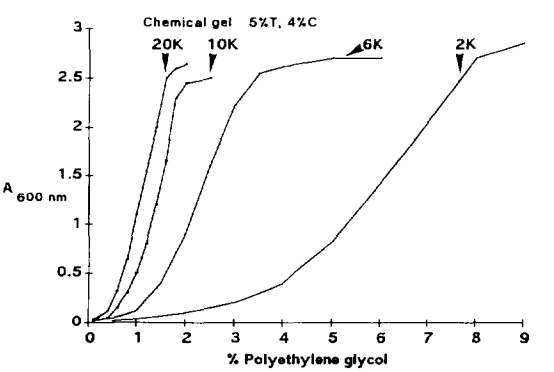


Fig. 3. Gel turbidity *vs.* type and percentage of "laterally aggregating" agent (PEG). 5% T, 4% C gels were polymerized in the presence of increasing amounts of PEG of $M_r$ 2000 (2K), 6000 (6K), 10 000 (10K) and 20 000 (20K). The gel opacity was read in a Varian spectrophotometer at 600 nm. The gels were cast in square-based (1 cm) cuvettes. From ref. 24.

solution was eliciting bundling of the growing polyacrylamide chains [24]. However, the phenomenon was unique in that, contrary to previous studies, this transition was obtained at regular percentages of cross-linker (in fact, barely 4% C). Additional studies suggested that the main phenomenon responsible for this bundling event was formation of inter-chain hydrogen bonds, occurring during the growth of the polymer and just prior to the cross-linking event. In fact, it was shown that urea and temperature (typical H-bond breakers), either alone or in association, would completely abolish the process whereas an agent such as tetramethylurea did not affect it [24].

The hypothesis of inter-chain H-bond formation was further corroborated by the following observations: (a) extremes of pH would quench the process; (b) the presence of monomeric ethylene glycol and even glycerol (both at concentrations >25%) would also hamper gel turbidity; and (c) production of mixed-monomer matrices (*e.g.*, acrylamide admixed with either N-methylacrylamide or N,N-dimethylacrylamide) also interfered with gel turbidity [25]. It remained to be demonstrated whether such highly turbid matrices had indeed a large pore size.

This was proved in two different ways. First, we found it possible to prepare gel samples by freeze-fracture and to dehydrate the matrix without bursting the membranaceous structure apart. When observing such specimens by scanning electron microscopy, it was found that, indeed, in matrices in which the phenomenon had plateaued (*e.g.*, in the presence of 2.5% $M_r$ 10 000 PEG) large fibre bundles (*ca.* 200–300 nm thick) were present, delimiting "holes" of equivalent diameter [24]. This was an impressive increment of pore size, of about two orders of magnitude compared with the same type of gel (5% T, 4% C) polymerized in the absence of PEG. An additional, direct proof came from the experiment in Fig. 4: the gels shown therein are unique, in that they demonstrate the possibility of creating a porosity gradient at a fixed concentration of monomers throughout, simply by preparing the gel in the presence of a gradient of "laterally aggregating" agent. It is seen that, in a conventional 6% T, 4% C matrix, large DNA fragments [21 000 base pairs (bp)] cannot even penetrate the gel meshwork and are trapped at the deposition site. However, when the same gel is polymerized in a 0.1–1% gradient of $M_r$ 10 000 PEG, the same 21 000 bp fragments penetrates

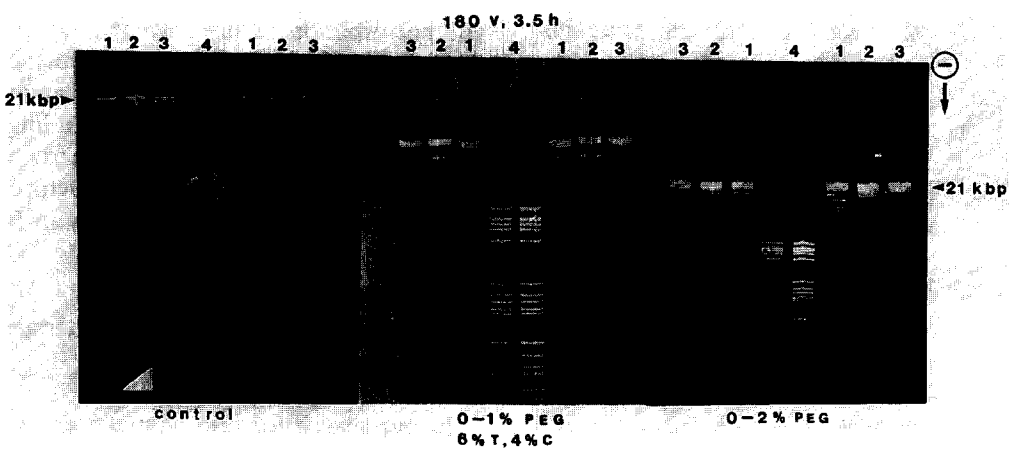


Fig. 4. Fractionation of DNA fragments in control and "laterally aggregated" gels. All gels were 6% T, 4% C in the absence (control, left) or presence of a linear gradient from 0 to 1% (central) or from 0 to 2% (right) of $M_r$ 10 000 PEG. All gels were run for 3.5 h at 180 V in Tris–borate–EDTA buffer (pH 8.3). Staining by dye intercalation with ethidium bromide. Note how the 21 000 bp fragment is trapped at the origin in control gels, whereas it migrates down 15% of the gel length in presence of the 0–1% PEG gradient (central gel) and 30% of the gel length in the 0–2% PEG gradient (right gel). Samples: 1 = $M_r$ marker III; 2 = $M_r$ marker II; 3 = $M_r$ marker I; 4 = $M_r$ marker V. From ref. 24.

15% down the gel length and, in a gel cast against a 0–2% gradient of $M_r$ 10 000 PEG, it migrates down 30% of the gel length (all other conditions, such as gel length, running time, temperature, voltage and buffer composition, being identical). We have additionally demonstrated that, in such matrices, one can focus immunoglobulins M ($M_r \approx 10^6$). It is well known that such large protein particles can be focused only in agarose matrices.

How can the pore size be manipulated in polyacrylamide matrices? According to the Ogston model [16]:

$$R = 1/\sqrt{4\pi nL}$$

where $R$ is the radius of a sphere which can be accommodated within the open spaces of a gel, $L$ is half the length of the gel fibre and $n$ is the number of fibres per $cm^3$ of gel volume. Note, however, that the above equation can only be applied for networks consisting of very long fibres having a negligible thickness, *i.e.*, with fibre radius $r = 0$). In our case, given the substantial thickness of the gel fibres in laterally aggregated gels, their radius has to be subtracted from the calculated value of the most frequent pore size population. Thus:

$$R = 1/\sqrt{4\pi nL} - r$$

There therefore appear to be two ways of accommodating larger objects in a gel network (thus increasing the pore size): either by reducing the fibre length or by decreasing the number of fibres per unit volume (or both). The procedure adopted by us is based on the strategy of drastically diminishing the number of fibres per unit gel volume by forming large bundles of chains. As PEG coordinates large amounts of water around its coil, it can be hypothesized that the perturbation of the solvent phase due to its presence could force the growing polyacrylamide strings to seek hydrogen bonding among themselves, rather than with the surrounding solvent. Once such aggregates are formed, they are stabilized in an irreversible structure by the cross-links, as shown schematically in Fig. 5.

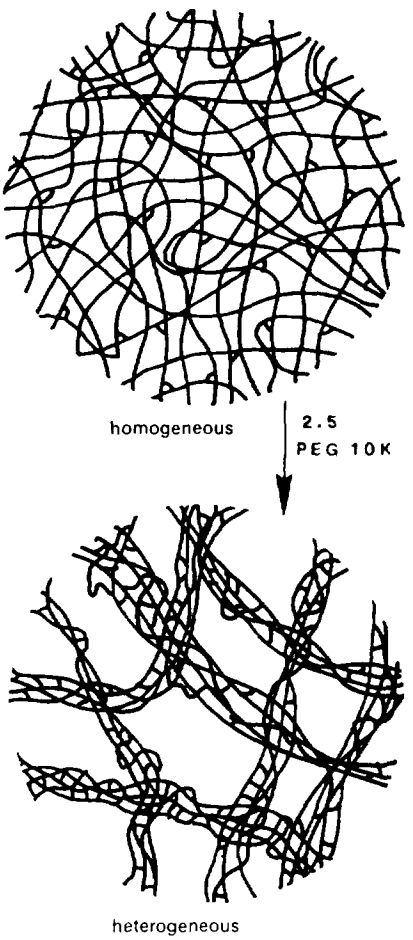


Fig. 5. Hypothetical model for "laterally aggregated" gels. The upper drawing could represent the structure of a control gel (a random meshwork of fibres). This gel is homogeneous in the sense that all fibres have random orientation in the three-dimensional space. The lower drawing could represent a gel polymerized in the presence of hydrophilic polymers. Owing to bundling of individual fibres (chain clustering), the average porosity is greatly increased. From ref. 24.

## 4. Macroporous polyacrylamides: temperature-induced aggregation

In our search for porous matrices, we noticed another phenomenon that could be correlated with the "lateral aggregation" event described above in the case of preformed polymers. We were trying to standardize a novel photopolymerization system, consisting of 100 $\mu M$ methylene blue in presence of a redox system, 1 m$M$ sodium toluenesulphinate (a reductant) and

50 $\mu M$ diphenyliodonium chloride (an oxidant) [26]. We noticed that, when photopolymerization was started at 2°C, the matrix formed was completely clear at the gelation point. However, if the polymerization was continued, after the gel point, at 2°C, the gel became progressively turbid, with the Tyndall effect plateauing after 50 min of reaction. In contrast if, after gelation at 2°C, the reaction was continued at 50°C, a fully transparent matrix was obtained. By plotting the turbidity *vs.* the polymerization temperature, a melting point at 28°C was obtained (see Fig. 6), suggesting that, by gelation at 2°C, the nascent chains formed clusters held together by hydrogen bonds, a process mimicking "lateral aggregation" as induced by gelling in the presence of exogenous polymers, such as PEG.

However, one puzzling phenomenon was also observed: if, after reaching the gel point at 2°C, the cassette was immediately placed at 50°C, the gel ripened by staying completely transparent. This could only be interpreted by assuming that, in the growing polymer, there would be a number of pendant, but still unreacted, double bonds. On maturation, these pendant bonds would be consumed. Therefore, if the reaction was started and continued for the whole time at 2°C, the nascent chains would first form clusters held together by H-bonds and subsequently lock such bundles in the three-dimensional space as

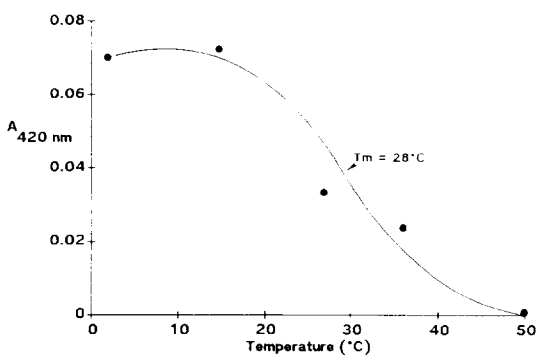


Fig. 6. Determination of the melting point ($T_m$) of the turbid gel phase on polymerization at different temperatures (2–50°C). The absorbance plateau values on gel ripening are plotted against the respective polymerization temperatures. The curve was traced by a sigmoidal curve-fitting program and $T_m$ was assessed at 28°C. From ref. 26.

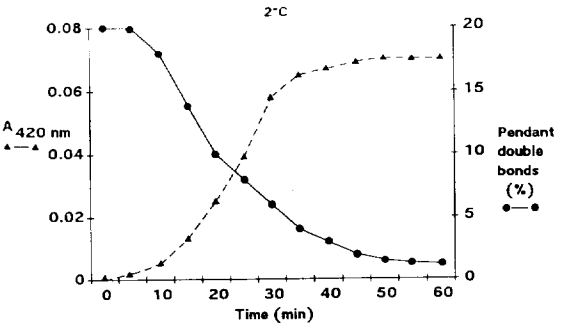


Fig. 7. Reaction kinetics of the pendant double bonds. After gelation, at the times indicated, ungrafted monomers and catalysts were extracted with methanol and the gel phase was titrated for unreacted double bonds by iodimetry. In parallel, the progress of the Tyndall effect (gel opacity) was monitored at 420 nm. From ref. 26.

the pendant double bonds in the chains were allowed progressively to react.

These two concomitant events are well illustrated in Fig. 7, which, to our knowledge, represents the first example of such a chain of events. On the basis of these observations, we have modified our model of Fig. 5 to account also for the additional constraints due to temperature. Basically, when no constraints are imposed on the growing polymer, the gel will form a domain of a random meshwork of fibres (as shown in the upper part of Fig. 5). However, if there are motion constraints, such as those caused by a preformed polymer in solution, chain bundles will form, producing a large-pore gel (Fig. 5, lower part). In the presence of another motion constraint, low temperatures, bundling also occurs, with the formation of large-pore matrices (Fig. 8).

## 5. Novel polymerization chemistry

We have recently described a unique photopolymerization system, consisting of a cationic dye (methylene blue, MB) and a redox couple (sodium toluenesulphinate, a reductant, and diphenyliodonium chloride, a mild oxidant) [27]. MB-driven catalysis offered highly reproducible gel points (180 ± 8 s at 30°C) and produced gels with better viscoelastic properties than equiva-

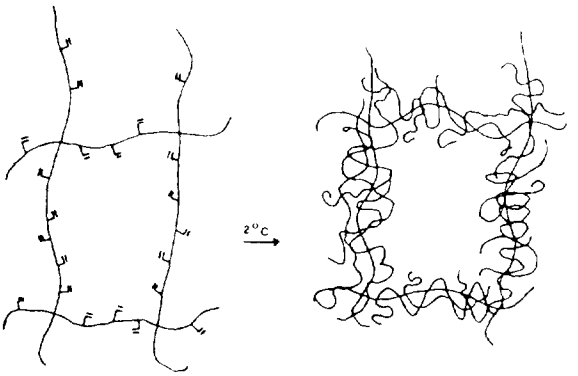

Fig. 8. Model of chain bundling at low temperatures. Left, hypothetical gel structure at the gel point, with several dangling unsaturated functionalities; right, at low temperature, hydrogen bonds would allow the formation of bundles of fibres, which the subsequent cross-linker reaction would "freeze" into space. From ref. 26.

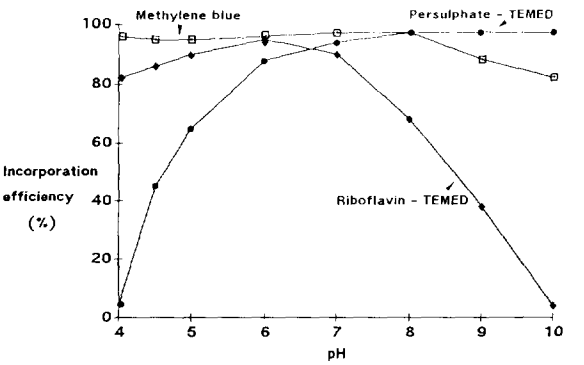


Fig. 9. Incorporation efficiencies of the systems peroxodisulphate–TEMED, riboflavin–TEMED and methylene blue (MB) *vs.* pH of the gelling solution. The conversion was assessed by eluting the ungrafted monomers from the gel phase, followed by separation and determination of free monomers by capillary zone electrophoresis. The molarity ratio of the monomers in the gel *vs.* the free liquid phase is taken to represent the incorporation efficiency. Note that, in the riboflavin system, optimum incorporation occurs only in the narrow pH range 6.2–6.5; in peroxodisulphate, the pH optimum is in the range 7.5–10, whereas in MB catalysis excellent conversion can be obtained in the entire pH range 4–10. After ref. 29.

lent gels chemically initiated with the standard redox couple, peroxodisulphate and TEMED [27]. In a subsequent theoretical study, it was found that an excellent conversion efficiency (in all instances >96% incorporation of monomers into the growing polymer) could be obtained, provided that the correct levels of dye and proper light intensity were used [28]. Paradoxically, if the levels of sensitizer or incident light intensities used were too high, the rate of dye consumption was found to be too high compared with monomer incorporation, so that dye depletion might occur prior to chain elongation and the reaction would then suddenly cease. However, when correctly used, photopolymerization was still found to give the best conversion efficiency among different initiation processes [28].

In a third paper of the series [29], another unique feature of MB-driven catalysis was discovered: a very high conversion efficiency was guaranteed by this system over the entire pH range 4–10. Conversely, riboflavin-driven polymerization offered a maximum only over a narrow pH range (6.2–6.5), the reaction slowly declining at acidic pH values and being strongly quenched at progressively alkaline values, until complete inhibition at pH 10. A specular behaviour could be demonstrated with peroxodisulphate–TEMED initiation: complete inhibition at pH 4, with the highest conversions in the pH range 7–10 (see Fig. 9). Two other systems investigated [ascorbic acid–iron(II) sulphate–hydrogen peroxide and peroxodisulphate–TEMED–hydrosulphite] exhibited only modest conversion efficiencies [29].

In a fourth paper in the series [26], MB catalysis was used for studying incorporation parameters of both monomers (acrylamide and Bis) in the proximity of a critical point, the gelation point ($p_c$). We exploited a unique property of such a gelling system, *i.e.*, the capability of essentially arresting the reaction by simply placing the gel cassettes in the dark. At $p_c$, assessment of monomer incorporation by capillary zone electrophoresis (CZE) gave a value of 50% for acrylamide and 80% for Bis. Such incorporation levels remained essentially unchanged in the temperature range 2–36°C. Interestingly, we found that, by initiating the polymerization at 2°C and continuing the process at this temperature, the gel became progressively

turbid, suggesting that the nascent chains formed clusters held together by hydrogen bonds, thus producing a "large-pore" gel (see Figs. 6–8 and the discussion on this phenomenon in the previous section).

In a further paper in the series [30], the efficiency of MB- *vs.* peroxodisulphate-driven catalysis was assessed in a series of aqueous–organic solvents [all in a 50:50 (v/v) ratio]: dimethyl sulphoxide, tetramethylurea, formamide and dimethylformamide, reported as useful disaggregating agents in a number of papers dealing with RNA and DNA separations (see ref. 31 for a review). In all instances, the peroxodisulphate-catalysed reaction was strongly quenched and even completely inhibited, whereas photopolymerization was essentially unaffected by any of these organic solvents. Conversely, in 8 *M* urea solutions, the peroxodisulphate-driven reaction was accelerated, boosting the monomer conversion to near completion (>98%), while leaving the photopolymerization process largely unaffected. Hence it would appear that photocatalysis with MB is a unique process, proceeding at an optimum rate under the most adverse conditions, completely insensitive to any kind of positive or negative effectors and able to ensure at least 95% monomer conversion under the standard conditions of a 1-h reaction time at room temperature.

In the final study in the series [32], we investigated the behaviour of both catalyst systems when polymerizing gels in the presence of detergents, as exemplified by electrophoresis in sodium dodecyl sulphate (SDS)-laden gels. The last technique is perhaps the most popular for assessing subunit structures of proteins and as a criterion of protein homogeneity. In addition to SDS electrophoresis, a host of other detergents have been proposed, especially for sample solubilization prior to two-dimensional maps. MB catalysis performed extremely well in the presence of various types of detergents (anionic, cationic, zwitterionic and neutral), ensuring in all instances conversion efficiencies >95%. Conversely, peroxodisulphate-driven polymerization was often quenched and in some instances completely inhibited by surfactants [32].

## 6. Novel monomer chemistry

Over the years, the pair of monomers that have attained the greatest popularity have been acrylamide coupled to a cross-linker, N,N′-methylenebisacrylamide (Bis) [19]. However, several defects of such a matrix have been noticed upon prolonged use. The most dramatic drawback is its instability at alkaline pH values: after an electrophoretic run (most electrokinetic separations occur at alkaline pH for both proteins and nucleic acids), the dangling amino bonds are partly hydrolysed, originating carboxylic groups, which remain covalently bound to the polymer, which is thus transformed into a polyacrylate. This phenomenon generates strong electrosmosis, with matrix swelling and considerable distortions. In practice, after only a single electrophoretic run, the polyacrylamide matrix cannot be reused. This strongly limits its use in large-scale projects, such as the sequencing of the human genome, where the availability of reusable matrices would greatly shorten the analysis time and allow rapid progress of such a project around the world. Stable matrices would be also useful in capillary zone electrophoresis (CZE), where the gel cannot be extruded from the capillary when partially hydrolysed or malfunctioning [13].

Another common problem is the limited range of molecular sizes that can be efficiently sieved by polyacrylamides. Such a porosity range encompasses pore sizes from a 2–3 to *ca.* 20–30 nm in highly diluted matrices [24,25] (see also the Introduction). This limits the use of polyacrylamides to protein separations, whereas agarose gels are today almost exclusively used for the separation of nucleic acid fragments. Highly porous polyacrylamide matrices would thus allow fractionation also of nucleic acids in some intervals of length (macroporous gels, as described above, now offer a valid alternative).

A third problem is linked to the use of the standard redox pair of catalysts, peroxodisulphate and TEMED. As this is a redox couple, it is able to oxidize many substances containing amino groups (from primary to tertiary), thus producing N-oxides. Such N-oxides, which re-

main in the gel even after discharging excess of peroxodisulphate to the anode, are able to oxidize proteins, especially the SH residues to disulphide bonds (–S–S–) [33] (a solution to this problem is now available with methylene blue photocatalysis, which is, by its nature, non-oxidizing) [27].

Some earlier patent applications addressed some of the problems described above and proposed different types of monomers. In one instance [34], Trisacryl [N-acryloyltris(hydroxymethyl)aminomethane, NAT] was advocated for producing hydrophilic, large-pore gels for electrophoresis. The Trisacryl monomer had in fact been proposed for chromatographic support media by Girot and Boschetti [35,36]. As will be shown later, this monomer, although strongly hydrophilic, suffers from its inherent instability, as it degrades with zero-order kinetics. Its use for, *e.g.*, reusable or long-term storage matrices cannot be clearly advocated.

In another patent application [37], acrylamido-sugars were proposed, such as N-acryloyl(or methacryloyl)-1-amino-1-deoxy-D-glucitol or the corresponding D-xylitol derivative. This class of acrylamido monomers, which certainly possess good hydrophilicity and a higher molecular mass than unsubstituted acrylamide, is also extremely unstable, as it degrades with zero-order kinetics and thus does not seem to be a valid alternative, just as poly(NAT) mentioned above [38]. In another application [39], a broad class of N-mono- and -disubstituted acrylamido monomers was proposed as electrophoretic support media, including some of the monomers mentioned above. However, out of this vast class of potential monomers, Shorr and Jain [39] enucleated (and commercialized) only two preferred mixtures, as follows (verbatim quotation): "in one preferred embodiment, the polymers are formed by cross-linking polymerization of N,N-dimethylacrylamide with ethylene glycol methacrylate. In another preferred embodiment, the polymers are formed by cross-linking polymerization of N,N-dimethylacrylamide and hydroxyethyl methacrylate with N,N-dimethylacrylamide". Also these formulations do not appear to be optimal. As shown later, N,N-dimethylacrylamide, and similar alkyl-substituted acrylamides, are too hydrophobic, while the various methacrylate cross-linkers are too prone to hydrolysis and are also hydrophobic [40]. As a result, the commercialized product containing these formulations (Hydrolink) has to contain detergents to help in solubilizing the monomers. The corresponding emulsion often flocculates. Needless to say, when the Hydrolink matrix is applied to protein separations, strong hydrophobic interactions, precipitation at the application site and smears are regularly experienced. These examples show that the problems formulated above, namely the design of new matrices possessing simultaneously a high hydrophilicity, a high resistance to hydrolysis and a larger pore size, have not been addressed properly and are far from being solved.

In the past, we addressed various problems connected with the polymerization of hydrophilic gels; in particular, the extent of conversion of monomers into the polymer phase was investigated as a function of temperature [41], amount and type of cross-linker [42] and type of catalyst [43]. We also described the problems connected with the preparation of highly porous gels by using high levels of cross-linker (typically, above 20% C) [8,21], especially when the latter was an allyl compound, *i.e.*, an effective inhibitor of gel polymerization [21]. We even ventured to describe two new monomers, acryloylmorpholine and bisacrylylpiperazine [44], believed to open up new horizons, as they permitted electrophoresis in aqueous–organic solvents. However, as it turned out, we were avoiding a key issue: how to obtain matrices exhibiting simultaneously high hydrophilicity and extreme resistance to alkaline hydrolysis, an impossible marriage, indeed. The answer to all these problems has now arrived, with the birth of a novel, unique monomer, combining high hydrophilicity with hydrolytic stability: N-acryloylaminoethoxyethanol (AAEE).

As shown in Fig. 10, the performance of AAEE as the free monomer is unique. Three types of behaviour can be distinguished here: a central group of four monomers, exhibiting first-order degradation kinetics; one monomer (Tris-

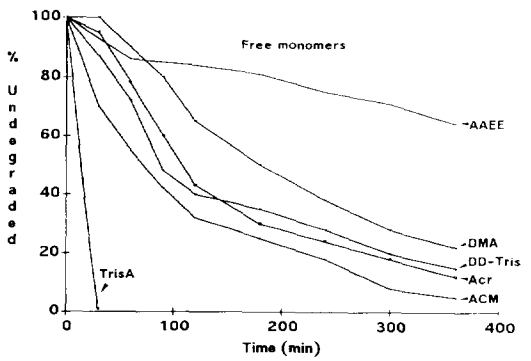


Fig. 10. Kinetics of hydrolysis of different monomers in free solution. Conditions: 0.1 *M* NaOH, 70°C, for up to 6 h. The extent of hydrolysis was assessed by neutralizing each hydrolysate and integrating the area of the undegraded monomer after separation in CZE. Abbreviations: Tris-A, trisacryl; ACM, acryloylmorpholine; Acr, acrylamide; dd-Tris, dideoxytrisacryl; DMA, dimethylacrylamide; AAEE, N-acryloylaminoethoxyethanol. From ref. 45.

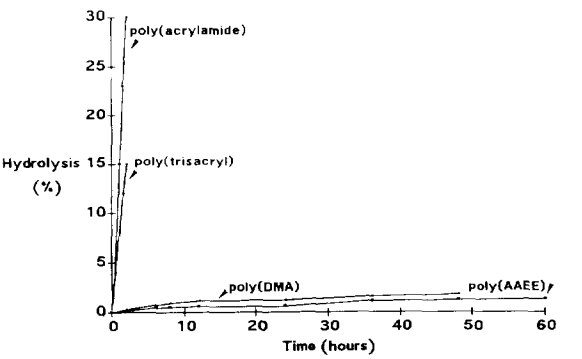


Fig. 11. Kinetics of hydrolysis of different monomers after incorporation in a gel matrix. Conditions: 0.1 *M* NaOH, 70°C, for up to 60 h. The extent of hydrolysis was assessed by measuring the equivalents of acrylic acid liberated in the polymer beads by frontal analysis. These equivalents were then transformed into the percentage of total amide groups hydrolyzed in the polymer. Abbreviations: poly(DMA), poly(dimethylacrylamide); poly(AAEE), poly(N-acryloylaminoethoxyethanol). Note that there is a 500-fold difference in reactivity between polyacrylamide and poly(AAEE). From ref. 45.

acryl) showing zero-order kinetics, suggesting intrinsic instability, and, at the opposite extreme, one monomer (AAEE) exhibiting extreme resistance to alkaline hydrolysis.

As, in previous work [39], we had demonstrated that the hydrolytic behaviour of some monomers could be dramatically altered when incorporated into the polymer matrix, we have further measured the degradation kinetics of such monomers in the gel phase. Fig. 11 summarizes the results obtained on mild alkaline hydrolysis (0.1 *M* NaOH, 70°C, up to 60 h). On this time scale, the behaviour of polyacrylamide, as compared with poly(AAEE) or poly(DMA), is dramatically different: whereas the former, after 2 h of incubation, is already degraded by 30%, the two latter matrices exhibit barely 0.07% hydrolysis. Hence there is a 500-fold difference between the susceptibility to degradation of the two classes of matrices. Even on prolonging the incubation to 60 h, poly(AAEE) shows only 1.22% amido groups hydrolysed.

Although we seem to have found at least two interesting monomers, from the point of view of resistance to hydrolysis under both acidic and alkaline conditions, there is another parameter to be assessed: the hydrophilicity of these novel compounds. A requirement for high hydrophilicity is fundamental for electrophoretic matrices, especially for protein fractionations, as hydrophobic interactions could ensue with decreasing hydrophilic properties of the monomers. A hydrophobicity scale has thus been established, by equilibrium partitioning in water–*n*-octanol phases. As shown in Fig. 12, Trisacryl lies at the extreme hydrophilic site, while dideoxy-Trisacryl shows an increment of hydrophobicity of about two orders of magnitude compared with the former. DD-Tris, in reality, cannot be used at all for producing a gel matrix, as it gives a gel so hydrophobic that it collapses, exudes water and is opaque. In practice, it appears that the partition coefficient of any monomer cannot be much higher than that of acrylamide (0.2), since a poly(DMA) matrix shows strong hydrophobic adsorption of proteins during isoelectric focusing. From this point of view, the novel monomer reported here (AAEE) seems to be unique, in that its partition coefficient is half that of acrylamide. Hence this monomer appears to be the only one, at present, able to couple a high hydrophilicity with an extreme resistance to hydrolysis.

A unique example of what this novel matrix

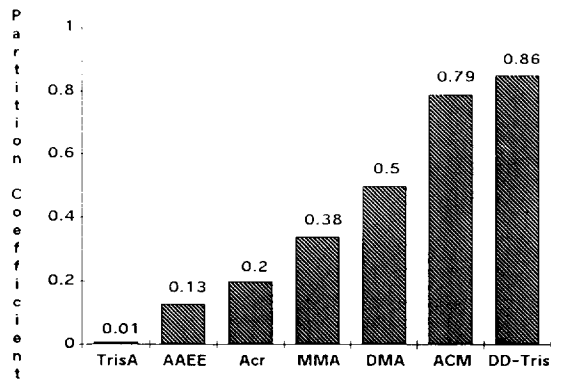


Fig. 12. Hydrophobicity scale of different acrylamide monomers. This scale was established by measuring the partition coefficient in water–*n*-octanol phases. The amount of each monomer remaining in the water phase was determined by CZE. Abbreviations: TrisA, Trisacryl; ACM, acryloylmorpholine; Acr, acrylamide; MMA, N-monomethylacrylamide; dd-Tris, dideoxy-Trisacryl; DMA, N-dimethylacrylamide; AAEE, N-acryloylaminoethoxyethanol. From ref. 45.

can do is shown in Fig. 13, which compares the behaviour of poly(AAEE) before and after mild hydrolytic conditions. The separation was performed by isoelectric focusing in immobilized pH gradients (IPG), known to be very sensitive to traces of acrylic acid in the polymer. The matrix, after polymerization, was cut into two halves,

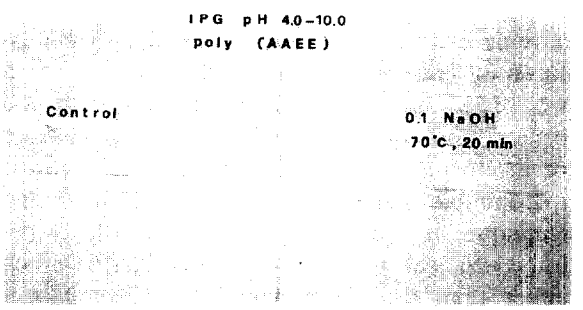


Fig. 13. Isoelectric focusing in immobilized pH gradients of haemoglobin in control and hydrolysed poly(AAEE) matrices. An IPG pH 5–10 interval was prepared in 6% T, 4% C poly(AAEE) matrices. A series was then subjected to hydrolysis in 0.1 *M* NaOH at 70°C for 20 min. Control and hydrolysed gels were then washed (three times in distilled water), dried and reswollen in 0.3% Ampholine pH 4–10. Focusing was carried out for 6 h at 3000 V, 10°C. Note the complete insensitivity of poly(AAEE) gels to the hydrolytic process. Conversely, it was impossible to focus protein samples in hydrolysed polyacrylamides, owing to the strongly acidic pH gradient developed in these matrices. From ref. 45.

one being used as a control and the other subjected to hydrolysis (20 min at 70°C in 0.1 *M* NaOH), followed by washing, drying and reswelling in 0.3% carrier ampholytes. As shown in Fig. 13, the poly(AAEE) matrix is completely insensitive to such a treatment, showing pH gradients essentially identical with those of control gels. In contrast, polyacrylamide gels (when usable; usually after this treatment they simply burst apart!) gave a long plateau between pH 4 and 5, whose inflection point (pH 4.6) was found to be the p*K* value of acrylic acid residues dangling from the polymer chains.

## 7. Polymer networks

We described in the Introduction the possibility of sieving macromolecules (both nucleic acids and proteins) in polymer networks, as envisaged long ago by De Gennes [10] and Bode [14,15]. While the technique did not gain momentum at its inception, it is now becoming popular with the advent of CZE. This is due to some simple reasons: first, the separation chamber (a capillary) is particularly well suited for holding *in situ* these viscous polymer solutions; second, although it is extremely difficult to fill a capillary with a cross-linked gel, while avoiding air bubbles and various undesirable phenomena, this procedure is facilitated in polymer networks; third, viscous polymer solutions (below a viscosity of 100 P s) [46] can be replenished in the capillary, thus providing a fresh environment for each separation. It is of interest to compare an entangled polymer solution mesh with a cross-linked gel mesh. The former has spatial properties, being temporarily transient and not in a permanent state [47]. A given entangled mesh persists at best up to the relaxation time of the polymer chains constituting the mesh. The residence time of analyte molecules in this mesh is controlled by the size and electrophoretic mobility of the analyte, the mesh size of the network and the imposed electric field strength.

According to either the Ogston model or the reptation mechanism, in order to achieve good

resolution, the relaxation time of the entangled polymer solution should be orders of magnitude greater than the residence time of the analyte molecule. In this manner, the mesh is maintained during transit of the analyte, so that the matrix serves as an effective sieving medium.

One interesting property of entangled polymer meshworks is shown in Fig. 14: it is seen that, no matter how large is the analyte size (in this instance a DNA stand), even when trapped in a polymer string, it can force it open and pass through. Hence one of the fundamental characteristics of polymer networks is that there is an almost infinite gradation of pore sizes: as the pores are dynamic, and not fixed in a rigid reticulum, any object, no matter how large, can force its way through the meshwork (thus a new migration mechanism has now been described, based on the concept of "constraint release") [48].

In electrophoresis in entangled polymer solutions, it has been predicted that, for a given high-molecular-mass polymer forming the network, the size of the largest DNA than can be separated increases roughly linearly with increasing viscosity of the solution [48]. One important practical implication of electrophoresis in polymer networks is that there is essentially no trapping or precipitation of the sample at the injection port (even in PCR-amplified fragments in the presence of large genomic material), a drawback always occurring in gel slabs, in the presence of a cross-linked matrix. In addition to the problems of instability of conventional polyacrylamide, owing to hydrolysis of pendant amido groups, this sample precipitation in the application wells is another important cause

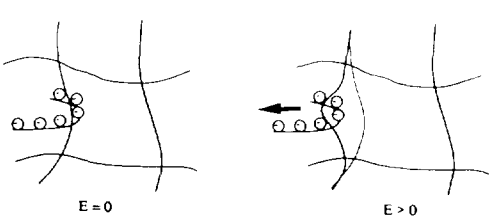


Fig. 14. Pictorial representation of the transient interaction of a DNA molecule with one of the strands constituting an entangled mesh. Note the forces acting on the strand, causing dissociation of the network. From ref. 47.

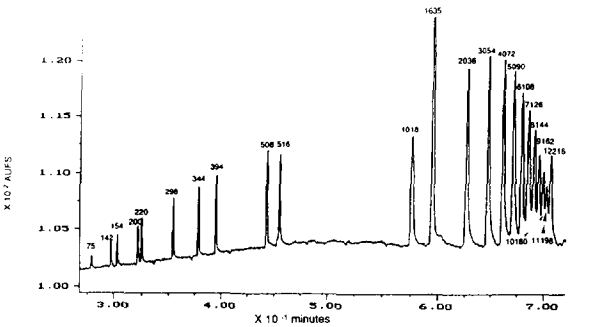


Fig. 15. CZE separation of the 1000 bp ladder. Conditions: 39 cm long capillary of 100 $\mu$m I.D., in 100 m*M* Tris–borate buffer (pH 8.2) and 2 m*M* EDTA, containing 10% T poly(AAEE) (at 0% C). Sample injection, 3 s at 4000 V; run, 4000 V at 8.8 $\mu$A; detection at 254 nm; Waters Quanta 4000 capillary unit with forced air cooling. The numbers on each peak represent the fragment length. From ref. 49.

which renders impractical reuse of a gel matrix, which for all practical purposes can be regarded as a disposable, single-shot column. Conversely, polymer networks [especially when utilizing poly(AAEE) matrices] allow the use of the same column for well over 50 sample injections [49].

A good example of the separation power of viscous polymer solutions is shown in Fig. 15, which displays the electropherogram of a sample of 1000 bp ladder in a coated [with poly(AAEE)] capillary, filled with a 10% T, 0% C poly(AAEE) solution in 100 m*M* Tris–borate buffer (pH 8.2) containing 2 m*M* EDTA. A few features are worth noting: (a) the great stability of the baseline, (b) the high sensitivity of detection, by which even a small fragment of 75 bp gives a clear peak and (c) the baseline resolution of most fragments, with a near-base resolution even for the large fragments, such as the 10 180 bp and the 11 198 bp species.

## 8. Conclusions

There has been marked progress in the last few years in polyacrylamide chemistry, which we can summarize thus: (a) the possibility of gelling "macroporous" matrices by exploiting different ways of lateral aggregations, polymer- and tem-

perature-induced; (b) the possibility of high-conversion gelling conditions, exploiting photopolymerization with methylene blue, MB catalysis, as an extra bonus, being devoided of oxidation power; (c) the discovery of a novel monomer, N-acryloylaminoethoxyethanol (AAEE), combining high hydrophilicity with extreme hydrolytic stability; and (d) the possibility of sieving in polymer networks, a most versatile "dynamic matrix" in capillary zone electrophoresis of macromolecules.

This is truly welcome, as electrophoresis up to the present has had little success in competing with chromatography. Just to give some examples of how the latter field has been growing: a recent survey has shown that, in Japan alone, during January 1988 to July 1992, a total of 337 patents have been issued to 125 enterprises, including national research laboratories and six individuals [50]. The largest number of patents issued to one company was 32 (Daice Industries, chiral columns), followed by Shimadzu (26, HPLC instruments), Hitachi (20, instruments), Showa Denko (16, polymeric columns), Mitsubishi Kasei (15, columns), Tosoh (14, columns and instruments), Mitsui Toatsu (13, columns) and Sumitomo Chemicals (12, columns). Majors [50] concluded (perhaps slightly ironically) that, "the future looks very bright for Japanese development of polymer column technology". Preparative chromatography of biomolecules also offers a great variety of methods [51,52], which reach a peak in affinity chromatography, where an incredible array of support media could be devised, tailored to essentially any type of macromolecule [53]. It is thus refreshing to see that, finally, electrophoresis is also moving to keep pace with modern times.

## 9. Abbreviations

| | |
|---|---|
| %C | g Bis/%T |
| %T | (g acrylamide + g Bis) per 100 ml of solution |
| $\langle a \rangle$ | pore radius |
| AAEE | N-acryloylaminoethoxyethanol |
| ACM | acryloylmorpholine |
| Acr | acrylamide |
| Bis | N,N′-methylenebisacrylamide |
| bp | base pairs |
| C | gel concentration (%, w/v) |
| CZE | capillary zone electrophoresis |
| $d$ | gel fibre or pore diameter |
| dd-Tris | dideoxy-Trisacryl |
| DMA | dimethylacrylamide |
| IPG | immobilized pH gradients |
| MB | methylene blue |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| $r$ | pore radius |
| $R_g$ | radius of gyration |
| TEMED | N,N,N′,N′-tetramethylethylenediamine |
| Tris-A | Trisacryl [N-acryloyltris(hydromethyl)aminomethane |

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