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Towards new formulations for polyacrylamide matrices, as investigated by capillary zone electrophoresis

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ABSTRACT

Several new aspects of polyacrylamide gel polymerization were investigated. First, a series of mono- and disubstituted acrylamide monomers were evaluated as potential candidates of a novel class of polyacrylamide matrices, exhibiting high hydrophilicity, high resistance to hydrolysis and larger pore size than conventional polyacrylamide gels. A series of cross-linkers were also assessed and their contributions to the gel stability and hydrophilicity evaluated. A novel method of photopolymerization is described, consisting of photoinitiating the reaction with methylene blue in the presence of a redox couple (sodium toluenesulphinate and diphenyliodonium chloride). The photobleaching curve gives direct information on the conversion efficiency of monomers into the growing polymer. In addition, photopolymerized gels have a better elastic modulus than peroxodisulphate-initiated gels. A unique correlation is described between the Phantom modulus and protein mobilities as a function of the percentage of cross-linker: a maximum of Phantom modulus corresponds to a minimum of mobility and both of these values occur at 5% of cross-linker. A novel method is described for producing extremely large-pore gels: polymerization in presence of a preformed, hydrophilic polymer (typically PEG 20 000 or PEG 10 000). In the presence of the latter polymers (up to 2–2.5%), "lateral-chain aggregation" occurs, with an extremely large expansion of pore size. For example, while a 5%T, 4%C gel typically has an average pore diameter of 5–6 nm, the same gel, in the presence of a "laterally aggregating agent" exhibits an average pore size of 500 nm.

INTRODUCTION

Polyacrylamide matrices, for separation in zone electrophoresis, were introduced in 1959 by Raymond and Weintraub [1] and further promoted for use in disc electrophoresis by Davis [2], Ornstein [3] and Hjertén [4]. Their popularity as electrophoretic supports stems from some fundamental properties, such as optical transparency, including the ultraviolet region, electrical neutrality, due to the absence of charged groups, and the possibility of synthesizing gels with a wide interval of porosities. The monomer that has attained the greatest popularity is acrylamide, coupled to a cross-linker, N,N'-methylene bisacrylamide [5]. However, several defects of such a matrix have been observed on

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prolonged use. The greatest drawback is its instability at alkaline pH: after an electrophoretic run (most electrokinetic separations occur at alkaline pH for both proteins and nucleic acids), the dangling amido bonds are partly hydrolysed, leading to carboxylic groups, which remain covalently bound to the polymer, which is thus transformed into a polyacrylate. This phenomenon generates strong electroendosmosis, with matrix swelling and considerable distortions. In practice, after only a single electrophoretic run, the polyacrylamide matrix cannot be re-used. This strongly limits its use in large-scale projects, such as the sequencing of the human genome, where the availability of re-usable matrices would greatly shorten the analysis time and allow for rapid progress of such a project around the world. Stable matrices would also be useful in capillary zone electrophoresis (CZE), as the capillary has to be discarded when the gel filling is partially hydrolysed or malfunctioning [6].

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 2–3 to *ca.* 20–30 nm in highly diluted matrices [7]. This limits the use of polyacrylamides to protein separations, whereas agarose gels are today almost exclusively used for separations of nucleic acid fragments. Highly porous polyacrylamide matrices would thus allow the fractionation also of nucleic acids into some intervals of length.

A third problem is linked to the use of the standard redox couple of catalysts: peroxodisulphate and N,N,N′,N′-tetramethylethylenediamine (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 remain 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–) [8].

Some earlier patent applications have addressed a few of the problems described above and have proposed different types of monomers. In one instance [9], Trisacryl [N-acryloyltris-(hydroxymethyl)aminomethane, NAT] has been 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 [10]. As will be shown in this paper, this monomer, although strongly hydrophilic, suffers from inherent instability, as it degrades with zero-order kinetics. Its use for, *e.g.*, re-usable or long-term storage matrices cannot be clearly advocated. In another patent application [11], acrylamido sugars have been 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, similarly to poly(NAT) mentioned above [12].

In another application [13], a broad class of N-mono- and -disubstituted acrylamido monomers have been proposed as electrophoretic support media, including some of the monomers mentioned above. However, of this large class of potential monomers, Shorr and Jain [13] have 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". These formulations also do not appear to be optimum. As will be shown in this paper, N,N-dimethylacrylamide, and similar alkyl-substituted acrylamides, are too hydrophobic, and the various methacrylate cross-linkers are too prone to hydrolysis and also are hydrophobic [14]. 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 have 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 [15], amount and type of cross-linker [16] and type of catalyst [17]. We also described the problems connected with preparation of highly porous gels by using high levels of cross-linker (typically, above 20%C) [18], especially when the latter was an allyl compound, *i.e.*, an effective inhibitor of gel polymerization [19]. We even ventured to describe two new monomers, acryloylmorpholine and bisacrylylpiperazine [20], believed to open up new horizons, as they permitted electrophoresis in aqueous–organic solvents. However, as it turned out, we (and we suspect many other scientists in the field) were avoiding a key issue, namely how to obtain matrices exhibiting simultaneously high hydrophilicity and extreme resistance to alkaline hydrolysis, an impossible marriage indeed.

In this paper, we address all three aspects mentioned above: the search for new monomers and cross-linkers, novel photopolymerization methods and gel polymerization conditions leading to extremely large pore sizes. In all these aspects, capillary zone electrophoresis (CZE) was instrumental in assessing the fate of the different monomers and their conversion efficiencies.

EXPERIMENTAL

Materials

Six monomers were analysed: acrylamide (Acr), N-methylacrylamide (MMA), N,N-dimethylacrylamide (DMA), N-acryloyltris-(hydroxymethyl)aminomethane (Trisacryl, Tris-A or NAT), N-acryloyldimethylhydroxymethylaminomethane (dideoxy-Trisacryl, DD-NAT) and N-acryloylmorpholine (ACM). Their synthesis has been described [3,12]. In addition, the following four cross-linkers were analysed: N,N′-methylenebisacrylamide (Bis), N,N′-(1,2-dihydroxyethylene)bisacrylamide (DHEBA), N,N′-diallyltartardiamide (DATD) and N,N′-bisacrylylcystamine (BAC). Gels were prepared by using acrylamide as a monofunctional monomer and any of the four cross-linkers. Acrylamide, TEMED, the four cross-linkers and ammonium peroxodisulphate were obtained from Bio-Rad Labs. (Richmond, CA, USA). Fused-silica capillaries (50 and 100 μm I.D., 370 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Methylene blue (puriss. DAB), toluene-4-sulphinic acid (sodium salt, anhydrous, purum, 98%), diphenyliodonium chloride (purum, 99%) and pK 4.6 acrylamido buffer, used as internal standard in CZE runs, were obtained from Fluka (Buchs, Switzerland). Polyethylene glycol (PEG) 2000, 6000 and 10 000 were purchased from Merck (Darmstadt, Germany) and PEG 20 000 from Baker (Deventer, Netherlands).

Capillary zone electrophoresis

CZE was performed on a Waters Quanta 4000 system (Millipore, Milford, MA, USA) in a 50 cm × 100 μm I.D. capillary from Polymicro Technologies. All runs were carried out at 25°C in 100 mM borate buffer (pH 9.0), either as such or with 50 mM sodium dodecyl sulphate (SDS) added (micellar electrokinetic chromatography) [21]. All runs were made in the cathodic direction at 5 kV and 35 μA. Samples were loaded for 10 s by the "hydrostatic injection" method. Sample zones were revealed at 214 nm.

Partition coefficient

In order to establish a hydrophobicity scale, the various acrylamido monomers were subjected to partitioning in water–1-octanol as described by Purcell *et al.* [22]. The partition coefficient P is defined as the ratio between the molarity of a given compound in the organic *vs.* the aqueous phase. Partitioning was performed as follows: each monomer was dissolved (50 mM) in water saturated with 1-octanol; 3.5 ml of this solution and 3.5 ml of 1-octanol were transferred into a separating funnel and shaken for 2 min. After decanting for 1 h, the aqueous phase

was collected and centrifuged for 75 min at 4000 *g*. All operations were performed at 25°C. The clarified solution was diluted to *ca.* 1 m*M*, internal standard (0.5 m*M* p*K* 9.3 Immobiline) added and the mixture analysed in CZE as described above. The decrease in the peak area in the aqueous phase allows the assessment of the amount of monomer remaining in that phase and thus, by difference, the amount dissolved in the organic phase.

Titration of acrylate groups in polyacrylamides by frontal analysis

In order to assess the amount of protolytic groups (acrylic acid) produced on extensive hydrolysis of the different types of polyacrylamides, the gels were cast not as continuous layers but as beads (with a concentration of 10%T and 8%C[a]) by emulsion polymerization [23]. The beads were extensively washed, dehydrated in methanol and dried *in vacuo*. A known amount of dry beads (from 0.25 to 1 g) was then reswollen in water and then subjected to different hydrolytic conditions: in 0.1 *M* NaOH at 70°C for up to 60 h and in 0.1 *M* HCl at 70°C for up to 12 h. After extensive washing in water (to negligible conductivity) the beads were loaded on a 1.6 cm diameter column to a bed height of *ca.* 10 cm. Titration was performed with 0.1 *M* HCl and the eluate pumped at a constant rate (1.5 ml/min, LKB peristaltic pump) through a micro-conductimetric cell (4-μl volume, Orion conductimeter, 10 mS full-scale). The signal was traced on a Kipp & Zonen recorder (20 mV full-scale, 10 mm/min chart speed). When the conductivity curve had reached a plateau (corresponding to the conductivity of the titrant, diluted by the dead volume of the resin), the titration was stopped and a solution, with half the titrant molarity, was injected directly into the conductimeter cell: this was necessary in order to measure the inflection point of the curve.

For calculating the total amount of protolytic groups (acrylic acid) generated on the resin by alkaline or acidic hydrolysis (C_{tot}), the following equation applies:

$$C_{tot} = [V/L(L_1 - L_0) - G_v]M_{tit}$$

where V is the volume of titrant utilized (in ml, as measured with a burette), L is the total length of the recorder tracing (in cm) for this V value, L_0 is the length of the recorder tracing corresponding to the dead volume of all connecting tubings, L_1 is the length of the recorder tracing (in cm) up to the inflection point (as measured when pumping in the titrant at half molarity), G_v is the total gel volume (in ml) and M_{tit} is the molarity of the titrant. In our case, the value of C_{tot} obtained is divided by the resin dry mass, so that our data are expressed in μequiv. per gram of dry beads. These data are finally converted into percentage hydrolysis with time (see Fig. 3A and B).

Alkaline hydrolysis of free monomers

All monomers were dissolved (20 m*M* each) in 0.1 *M* NaOH and incubated at 70°C for up to 6 h. At given time intervals (see the relevant figures) aliquots were collected and diluted (to *ca.* 1 m*M*) in 0.1 *M* sodium borate buffer (pH 9.0) containing 50 m*M* SDS. After adding 2 m*M* Immobiline p*K* 4.6 as internal standard, the samples were analysed by CZE as described above. The decrease in the peak area, as compared with a reference, non-hydrolysed sample, was used for calculating the rate of hydrolysis.

Photopolymerization

All experiments were performed in 100 m*M* Tris–phosphate buffer (pH 8.2), in general using a monomer mixture of 6%T, 4%C. As a routine, the solutions were first degassed with a water pump for 10 min and then bubbled with argon for an additional 10 min. Stock solutions of catalysts were prepared in distilled water as follows: 2 m*M* methylene blue, 20 m*M* sodium toluenesulphinate (reducer) and 1 m*M* diphenyliodonium chloride (oxidizer). They were kept refrigerated in the dark and used for only one working week (except for the dye, which we have used for up to seven months with no decrease in efficiency). Under standard conditions, the gels were made to contain 100 μM dye, 1 m*M* reducer and 50 μM oxidizer. Photopolymerization was activated by placing the

[a] C = g Bis/% T; T = (g acrylamide + g Bis)/100 ml solution.

cassette between two light boxes (neon tubes, 12 W each) at a 10-cm distance from each source. Completion of reaction was evident by photobleaching.

Assessment of photobleaching

Photobleaching was measured by performing polymerization in plastic cuvettes of a spectrophotometer (1-cm light path) and covering the liquid surface with a layer of light paraffin oil (so as to avoid oxygen adsorption). At regular time intervals, the cuvette was quickly placed inside the spectrophotometer and the absorbance decreases read at 600 nm. In photofading experiments utilizing gel layer thicknesses from 0.5 to 2 mm, the gel cassette was assembled with two microscope glasses, kept apart by appropriate spacers, as described previously [18]. These cassettes were used as both polymerization containers and cuvettes for spectrophotometric readings.

Other types of polymerization

Gels were also polymerized with peroxodisulphate and TEMED (1 μl of 40% peroxodisulphate and 1 μl of pure TEMED per millilitre of gelling solution) or with riboflavin 5′-phosphate ($1.16 \cdot 10^{-5}$ mmol/ml). In some experiments, peroxodisulphate–TEMED-initiated gels were polymerized in the presence of a "laterally aggregating agent" (2.5% PEG 10 000) to produce macroporous structures.

Spectrophotometric reading

The turbidity of "laterally aggregated" gels was studied with a Varian (Palo Alto, CA, USA) DMS-90 UV–Vis spectrophotometer at 600 nm against a water blank. All gels were prepared at 5%T, 4%C matrices in the presence of variable amounts of preformed linear polymers, as follows: for the PEG series the concentrations added ranged from 0.1 up to 25% (w/v), for polyvinylpyrrolidone (PVP) from 0.2 up to 15% (w/v) for hydroxymethyl cellulose (HMC) of M_r 10^6 from 0.01 up to 0.1% (w/v). To all solutions were added 1 μl/ml of TEMED and 1 μl/ml of 40% ammonium peroxodisulphate. Each sample was run in triplicate in plastic cuvettes and was allowed to polymerize for 1 h at room temperature.

Electron microscopy

The following procedure proved to be optimum for sample preparation for the scanning electron microscope. The polymerized gels (as prepared in a spectrophotometric cuvette, in the presence of increasing amounts of PEG 10 000 from 0 up to 2.5%) are first fractured into several segments (not by cutting, but by breaking them), which are then equilibrated for 2 days in 2.5% PEG 10 000 (*i.e.*, the plateau concentration giving maximum turbidity and thus maximum chain aggregation). The fragments are quickly frozen in liquid nitrogen and immediately lyophilized without allowing melting during the entire process. It was found that, in the absence of the PEG 10 000 equilibration step, all samples containing either no PEG or smaller amounts showed variable degrees of disintegration of the gel structure during the various sample manipulations prior to microscopic observations. The samples were then spread with a gold–palladium thin layer in an Edwards 306 metallizer (Edwards High Vacuum, Crawley, UK) and then observed with a Stereoscan 250 scanning electron microscope (SEM) from Cambridge Scientific Instruments (Cambridge, UK) at 20 kV. Photographs were taken with a Kodak TRI-X PAN 120 film.

Elastic measurements

For elastic measurements the gel matrices were polymerized at room temperature in vertically placed rectangular glass cassettes of 10 mm thickness and 125 × 125 mm size. Photoinitiation was carried out by illuminating the chamber through one of the wide sidewalls by a four-tube lamp situated 10 cm from the chamber. At fixed time points the illumination was stopped, several (usually nine) cylindrical samples of 25 mm diameter were cut from the gel slabs and subjected to measurements with a dynamometer from Instron Engineering (Canton, MA, USA). A pulling force of 10 N full-scale was selected, and the traction was stopped at 3 N, at which point the compressed gel was left to expand. The experimental points plotted in Fig. 5 are statisti-

cal data for the series. Note that, as the gels dry quickly, the matrices were prepared immediately before the measurements and kept in a closed box during the experiment, and each sample was cut just before being subjected to measurements.

RESULTS

Properties of novel monomers and cross-linkers

For investigating several parameters of the various monomers, such as hydrolytic stability, partition coefficient and incorporation efficiency, we have standardized a separation and quantification method based on micellar electrokinetic chromatography. Fig. 1 gives a typical example of such separations: when investigating the incorporation efficiency of monomers and cross-linkers, the gel extracts were analysed by CZE in 50 m*M* SDS and revealed at 214 nm. This method allowed a clear separation of acrylamide from bisacrylamide, based on the different partition coefficients in the SDS micelle. The p*K* 4.6 Immobiline, admixed to the sample prior to injection, was used as an internal standard for quantification purposes.

Based on the results obtained by CZE, we established a degradation scale of the different monomers, as shown in Fig. 2A: it is seen that whereas almost all acrylamido derivatives exhibit first-order degradation kinetics, one of them

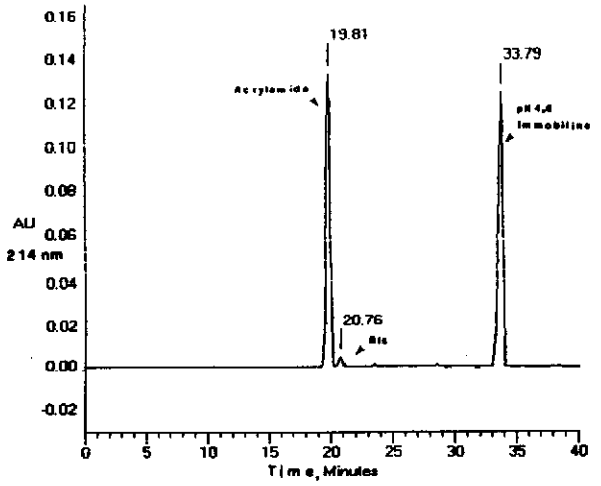

Fig. 1. Representative CZE analysis of ungrafted monomers. After eluting from the gel and adding the internal standard (2 m*M* p*K* 4.6 Immobiline), the monomer mixture was analysed by CZE in a 50 cm × 75 μm I.D. fused-silica capillary at 4000 V, 31 μA and room temperature. The buffer was 100 m*M* Tris–borate–50 m*M* SDS (pH 9.0). The sample was injected by gravity for 10 s. Detection was at 214 nm. The numbers on each peak represent the transit time.

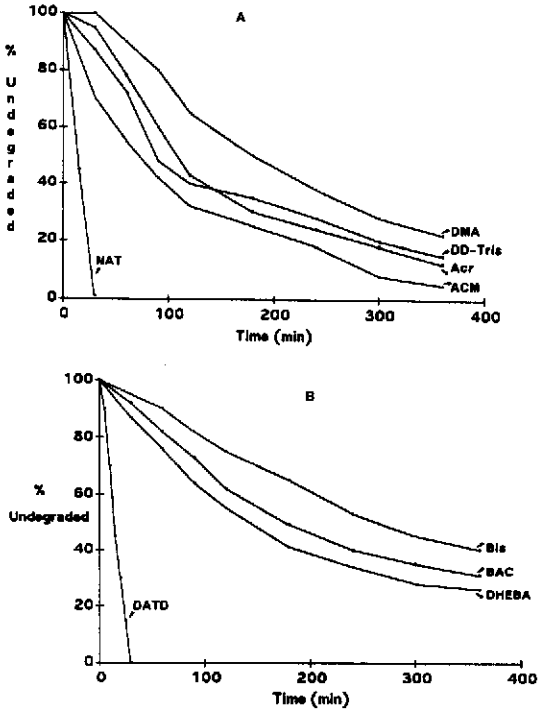

Fig. 2. Kinetics of hydrolysis of different acrylamide monomers (A) and cross-linkers (B). Hydrolysis has been performed in 0.1 *M* NaOH at 70°C for the times indicated. The amounts were assessed by harvesting triplicates at each point, neutralizing and injecting into the CZE instrument (Waters Quanta 4000). Conditions: 100 m*M* borate–NaOH buffer (pH 9.0), 15 kV, 86 μA at 25°C. Uncoated fused-silica capillary (50 cm × 75 μm I.D.). (A) Hydrolysis rate of different monomers, as follows: NAT = Trisacryl; Acr = acrylamide; MMA = monomethylacrylamide; DMA = N,N-dimethylacrylamide; ACM = acryloylmorpholine; DD-NAT = dideoxy-Trisacryl. Note that whereas all other monomers exhibit first-order kinetics, NAT follows zero-order degradation kinetics. (B) kinetics of hydrolysis of different cross-linkers. Abbreviations: Bis = N,N'-methylenebisacrylamide; DHEBA = N,N'-(1,2-dihydroxyethylene)bisacrylamide; DATD = N,N'-diallyltartardiamide; BAC = N,N'-bisacrylylcystamine. Note that whereas all other cross-linkers exhibit first-order kinetics, DATD follows zero-order degradation kinetics.

(Trisacryl) shows zero-order kinetics, suggesting that such a monomer is intrinsically unstable. Similar results were obtained with the four different cross-linkers (Fig. 2B): whereas most of them exhibit first-order degradation kinetics, as expected, one of them, DATD, displays a zero-order degradation process, suggesting that such a monomer, just like Trisacryl, is intrinsically unstable.

A unique picture emerges when the stability of the same monomers is analysed not as free species, but incorporated into the polymer network. For this purpose, all monomers were cross-linked with Bis and polymerized into beads, subjected to hydrolysis and then titrated by frontal analysis (this titration, being a chromatographic process, can only be performed with pearls, not with continuous beds). Fig. 3A shows the degradation kinetics of the monomers into the polymer phase, on alkaline degradation. It is now seen that poly(DMA) is extremely resistant to hydrolysis, as compared with both a polyacrylamide and a poly(Trisacryl). Whereas polyacrylamide, after 2 h of hydrolysis, is already degraded 30%, poly(DMA) exhibits only 0.07% hydrolysis. Hence there is a 500-fold difference between the susceptibility to degradation of the two classes of matrices. Even when prolonging the incubation to 60 h, poly(DMA) shows only 1.22% amido groups hydrolysed. Such a unique behaviour prompted an investigation of the hydrolysis rate also under acidic conditions. As shown in Fig. 3B, the hydrolysis rates in an acidic environment are accelerated by a factor of three compared with analogous conditions in NaOH. In any event, the excellent resistance to hydrolysis of poly(DMA) is confirmed even under acidic conditions.

We next established a hydrophobicity scale, based on partitioning in water–1-octanol phases and determining the remaining concentration in the aqueous phase by CZE. Table I summarizes the results for all the monomers and cross-linkers investigated. It is seen that the P values span all ranges, from extreme hydrophilicity (for Trisacryl $P = 0.01$) to extreme hydrophobicity (for BAC $P = 10$). Fortunately, most values lie in the range $P = 0.2$–0.4, *i.e.*, acceptable values for still producing hydrophilic matrices.

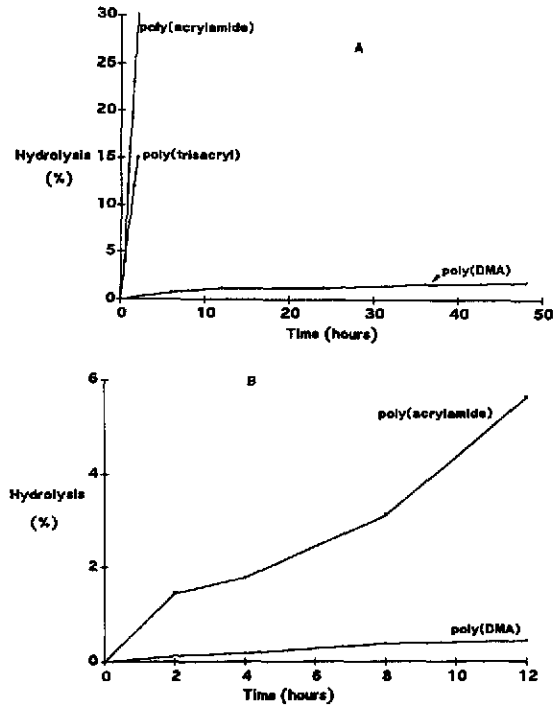

Fig. 3. Hydrolysis kinetics of different monomers after incorporation in a gel matrix. The extent of hydrolysis was assessed by measuring the equivalents of acrylic acid liberated in the polymer beads by frontal analysis. These equivalents were converted into the percentage of total amide groups hydrolysed in the polymer. (A) hydrolysis in 0.1 *M* NaOH at 70°C for up to 60 h. Poly(DMA) = polydimethylacrylamide. Note that there is a 500-fold difference in reactivity between polyacrylamide and poly(DMA). (B) Hydrolysis kinetics of poly(DMA) and polyacrylamide under acidic conditions (70°C, 0.1 *M* HCl) for up to 12 h.

Photopolymerization

Another important aspect of polyacrylamide chemistry is the correct choice of the catalyst system. We have recently found that chemical polymerization with the redox couple peroxodisulphate–TEMED is oxidizing, as it produces N-oxide species on all amino groups present in solution. Such N-oxides, during the electrophoretic analysis, can easily oxidize free –SH groups in proteins migrating through the gel phase [24]. We therefore reinvestigated photopolymerization, especially with riboflavin, and found that photoinitiation indeed occurs without concomitant oxidation [8,25].

TABLE I

PARTITION COEFFICIENTS OF MONOMERS AND CROSS-LINKERS

Compound	Partition coefficient (P)
Monomers	
Trisacryl	0.01
Acrylamide	0.2
Monomethylacrylamide	0.38
Dimethylacrylamide	0.5
Acryloylmorpholine	0.78
Dideoxy-Trisacryl	0.896
Cross-linkers	
DATD	0.18
DHEBA	0.27
Bis	0.82
BAC	10

Another catalyst system that we are currently evaluating is photoinitiation with methylene blue, coupled to the redox system toluenesulphinate–diphenyliodonium chloride. This system has some unique properties, as shown below. First, we found that the monitoring of the rate of photobleaching (*i.e.*, dye destruction during polymerization under light irradiation) gives a precise indication of the extent of conversion of monomers into the polymer phase. This is shown in Fig. 4: photobleaching was assessed by transferring the polymerization cassette (made of two microscope slides with a 0.5 mm gasket as spacer) into the spectrophotometer at the times indicated, and conversion efficiency was measured by extracting the gel and determining the ungrafted monomers by CZE. It is seen that, with this catalyst system, 70% conversion is obtained after 5 min of irradiation and >95% conversion is achieved after 50 min, when the dye is almost completely bleached. Hence the measurement of photobleaching appears to be a simple and reliable method for assessing the rate of polymer formation.

Gels obtained by photopolymerization also exhibit unique viscoelastic properties: as shown in Fig. 5, the elastic modulus of methylene blue-polymerized gels is better than that of peroxodisulphate gels, which in turn is considerably higher than that of peroxodisulphate gels in the

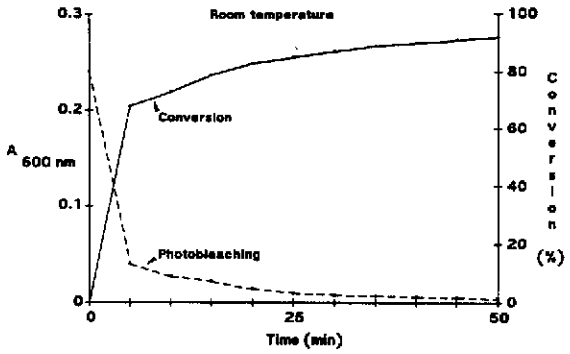

Fig. 4. Correlation between dye photobleaching and conversion efficiency. Photobleaching was assessed spectrophotometrically. For the incorporation efficiency, aliquots were harvested at 5-min intervals along the illumination curve, the monomers extracted, separated by CZE and determined as in Fig. 1. Note the extremely high incorporation efficiency in the first few minutes of illumination (70% in only 5 min), followed by a very slow rate of monomer uptake. Conversions >95% are ensured on illumination for 1 h in gel thicknesses up to 1 mm.

case of lateral aggregation. It should be noted that there is no contradiction between Figs. 4 and 5. In the former, we stated that 70% conversion was obtained in only 5 min. In Fig. 5, it is seen that the elastic modulus (which also is an indicator of the extent of conversion) reaches a plateau in 4–5 h for methylene blue, and in only 1 h for peroxodisulphate. It should be remembered that the conversion rate, in all

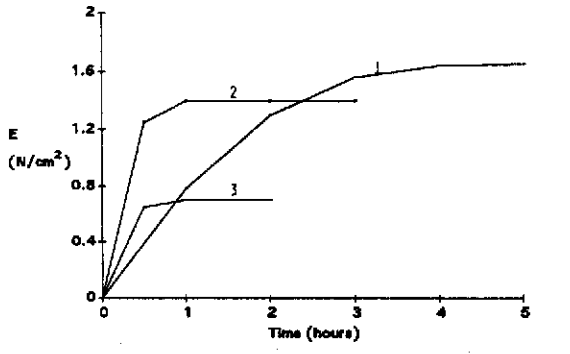

Fig. 5. Dependence of the elastic modulus (E, in N/cm^2) on the polymerization time in the case of methylene blue polymerization (1), peroxodisulphate (2) and peroxodisulphate in the presence of laterally aggregating agents (3). In the last instance the gel was chemically polymerized in the presence of 2% PEG 10 000. Note that although a plateau is reached at a slower rate, the best elastic properties are exhibited in the case of photopolymerization.

photopolymerization processes, is thickness dependent (which, of course, is not the case in peroxodisulphate-driven polymerization), as the outer liquid strata in the gel cassette absorb much of the incident light. In order to use the dynamometer, we were forced to adopt a 1-cm layer thickness, as opposed to 0.5 mm in Fig. 4.

We next investigated the Phantom modulus of methylene blue gels as a function of the percentage of cross-linker and correlated it with protein migration rates in the gel phase. As shown in Fig. 6, the Phantom modulus (expressed in N/mm^2) reaches a maximum at 5% cross-linker; conversely the migration velocity of proteins (expressed in mm/min) reaches a minimum at the same 5% cross-linking value. This is a unique observation and fully confirms the data first proposed by Fawcett and Morris in 1966 [26] that, for any family of %T gels, a minimum of porosity is always found at 5% cross-linker. These data also suggest another important correlation: a good "elastic body" (at least with polyacrylamides) is also a matrix exhibiting a minimum of porosity.

Another unique aspect of photoinitiation is illustrated in Fig. 7. It is well known that, in peroxodisulphate initiation, the polymerization efficiency decreases at acidic pH values (although the conversion had not been assessed before). In

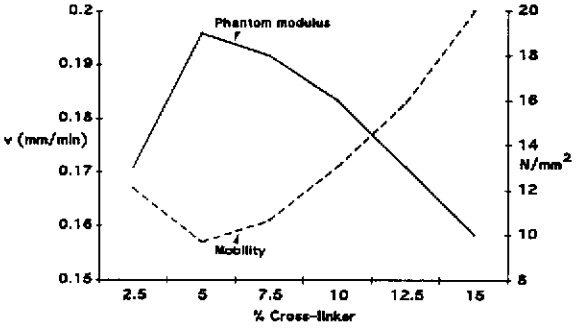

Fig. 6. Comparison between the gel elastic properties (Phantom modulus) and porosity as a function of percentage of cross-linker at constant %T. The gel porosity was assessed by measurement of the velocity of a protein marker (human serum albumin). The Phantom modulus data were measured with a dynamometer. The values on the scale of elastic measurements (in N/mm^2) should be multiplied by 10^3. Note that the minimum of mobility and the maximum of elasticity coincide at 5%C.

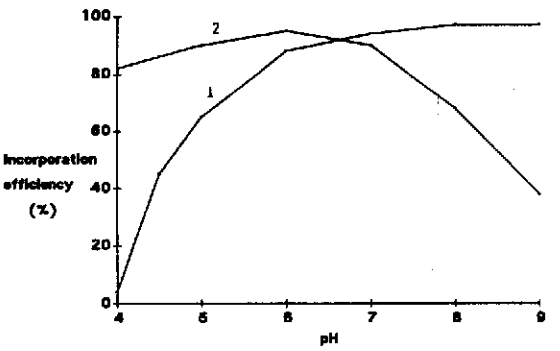

Fig. 7. Conversion efficiencies *vs.* pH of the gel solution in the cases of peroxodisulphate (1) polymerization and riboflavin (2) catalysis. The incorporation efficiencies were assessed in 5%T, 4%C gels after a standard polymerization time of 1 h at room temperature by extracting the ungrafted monomers and determining them in a CZE run (see Fig. 1). All solutions were prepared as follows: in 100 m*M* acetate buffer (pH 4 and 5); in 100 m*M* phosphate buffer (pH 6 and 7); in 100 m*M* Tris–chloride buffer (pH 8 and 9). All the various pH values were readjusted to the corrected pH after addition of TEMED (which usually increased the solution pH by *ca.* 0.5).

Fig. 7, it is shown that a plateau is reached in the pH range 7–9 (with maximum incorporation efficiency of *ca.* 95%), but that incorporation decreases steadily with decreasing pH until, at pH 4.0, no gelation occurs. An opposite behaviour is shown in riboflavin catalysis: the efficiency decreases substantially at alkaline pH values, with maximum incorporation efficiency around pH 6.0–6.4. However, in the entire pH 4–7 interval, gelation still occurs and the incorporation efficiency is still around 85–90% even at adverse pH values, such as pH 4 and 5. This offers a new insight into polyacrylamide catalysis: by selecting the proper catalyst (riboflavin in the pH range 4–6.5 and peroxodisulphate in the pH range 6.5–9), one can ensure satisfactory conversion efficiency throughout the entire operational pH interval. This is a novel finding not reported previously.

Laterally aggregated gels

This is a novel aspect of polyacrylamide chemistry, which we have recently discovered [7]. We shall highlight here some of the results obtained. It should be emphasized that, in the classic Ornstein–Davis system [2,3], the only

way to obtain highly porous polyacrylamide matrices was to increase dramatically the amount of cross-linker (%C) at a fixed and low total amount of monomers (%T). As demonstrated by Righetti *et al.* [18], 3%T gels, with up to 60%C cross-linking can reach average pore diameters as high as 500–600 nm. However, such gels are of no practical interest as they are highly hydrophobic and exude water, so that they represent a hazard (electric short-circuits) in open-face gel slab electrophoresis. We have recently found that, if polymerization is conducted in presence of a preformed, hydrophilic polymer in solution, a unique phenomenon occurs, which we have termed "lateral chain aggregation", resulting in a very large increase in pore size at normal values of %T and %C, *e.g.*, 5–6%T and 4–5%C. The process can be monitored by an increased gel turbidity as "lateral chain aggregation" progresses, as shown in Fig. 8. A typical polymer inducing such aggregation is polyethylene glycol (PEG), with an efficiency correlated with the mean molecular mass: a PEG 20 000 polymer drives the full transition (plateau level) already at 1.5% in solution, whereas *ca.* 10% PEG 2000 is needed for a similar transition (Fig. 8).

In Fig. 9, we have depicted what we think could happen during such a unique polymerization process: in the absence of PEG, the gel is truly a "random meshwork of fibres", as previous literature data suggest. This would give a

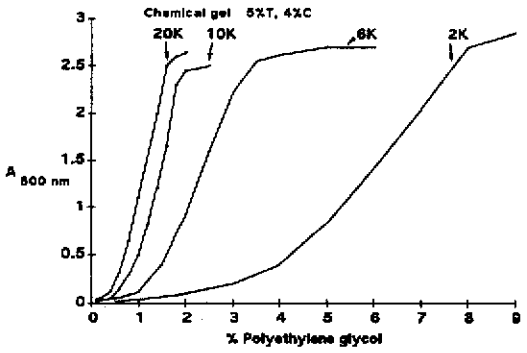

Fig. 8. Gel turbidity *vs.* type and percentage of laterally aggregating agents. 5%T, 4%C gels were polymerized in the presence of increasing amounts of PEG 2000 (2K), 6000 (6K), 10 000 (10K) and 20 000 (20K). The gel opacity was read on a Varian spectrophotometer at 600 nm.

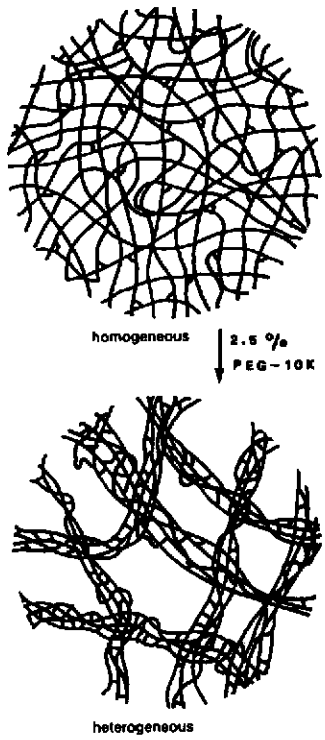

Fig. 9. Hypothetical model for "laterally aggregated" gels. The top drawing could represent the structure of a control gel (a random meshwork of fibres). This gel is homogeneous in the sense that all fibres have random orientations in the three-dimensional space. The bottom drawing could represent a gel polymerized in the presence of hydrophilic polymers. Owing to bundling of individual fibres (chain clustering), the average porosity is greatly increased.

three-dimensional structure resembling the "homogeneous" gel at the top of Fig. 9, consistent with a minimum of porosity. Conversely, in the presence of PEG, bundling or "lateral chain aggregation" occurs, resulting in a matrix with a much increased pore size (Fig. 9, bottom). Note that, in some ways, such a structure could resemble that of agaroses, known to form helices and super-helices (aggregates or pillars of 7–11 helices) on gelation. How can PEG [and other hydrophilic polymers, such as polyvinylpyrrolidone, poly(vinyl alcohol) and hydroxymethylcellulose] induce such a transition? We believe it to be mostly due to inter-chain hydrogen bonding, as such a process is greatly inhibited by the presence of 8 *M* urea in solution and by high temperatures. As PEG is a highly

hydrophilic molecule (coordinating up to 1.5 molecules of water per oxygen residue), as the nascent polyacrylamide strings grow in solution they tend to become hydrogen bonded among themselves, owing to the water-sequestering action of PEG. Once the cross-linking event has occurred, the "bundles" of chains are then irreversibly structured in the gel three-dimensional structure.

That our model in Fig. 9 might not be too far from reality is demonstrated by Fig. 10: by freeze-fracture, scanning electron microscopy, we can observe a smooth, apparently non-porous structure in a regular, control polyacrylamide (6%T, 4%C; Fig. 10A). In the presence of 0.8% PEG 10 000 in solution, a pitted and irregular surface appears (Fig. 10B). When the level is increased to 2% and 2.5% PEG (Fig. 10C and D, respectively), two phenomena are evidenced: fibres of 200–300 nm diameter appear, delimiting "holes" of *ca.* 500 nm diameter. The presence of such large bundles explains the light-scattering properties of such gels. However, in contrast to highly cross-linked gels, which reach similar porosities but are totally useless (at least for electrokinetic processes), the present gels appear to be excellent for electrophoretic fractionation. We have run, in such matrices, double-stranded DNAs up to 22 000 base pairs (bp), a size that cannot even penetrate a regular 6%T, 4%C gel (unpublished work). Moreover, in such highly porous matrices, focusing (including immobilized pH gradients) occurs in a fraction of the time needed in conventional matrices. Thus we believe that such a novel matrix will open up unique possibilities in electrophoretic separations exploiting polyacrylamide matrices.

DISCUSSION

Monomer chemistry

For at least 15 years our laboratory has been connected with the study of polyacrylamide gels and the development of new matrices. The first impulse came from an invitation by NASA (Dr. R. Snyder, Huntsville, AL, USA) in 1979, at that time strongly interested in developing space electrophoresis. We set out to measure the maximum pore size that could be produced in agarose and polyacrylamides under earth gravity. We derived an empirical equation, linking the pore

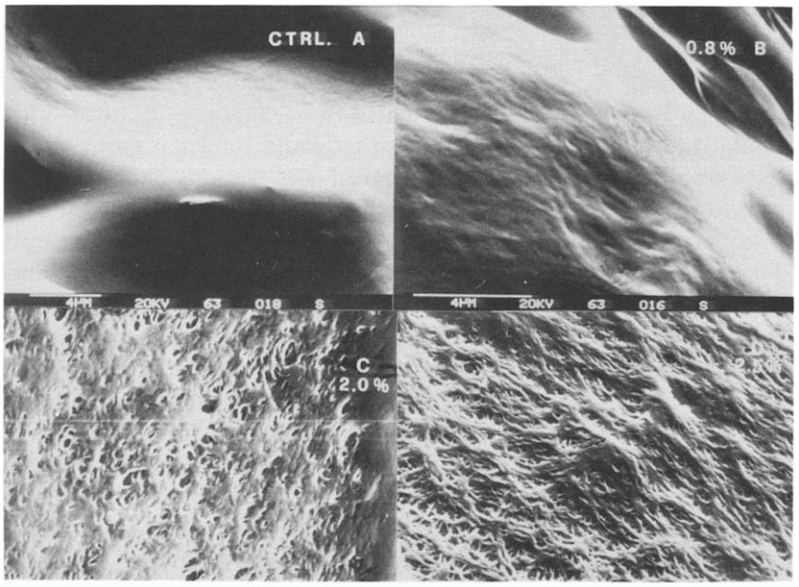

Fig. 10. Scanning electron micrographs of (A) control and (B–D) "laterally aggregated" gels. (B) 5%T, 4%C gel in the presence of 0.5% PEG 10 000; (C) same, but polymerized after adding 2% PEG 10 000; (D) same, but with 2.5% PEG 10 000 added. Note the smooth surface of the control gel, with no visible pores, and the progressive opening of the pore sizes in (C) and (D). In the last gel (D) the surface is seen to be made by regular bundles of 2–300 nm thickness, delimiting "holes" of *ca.* 0.5 μm diameter.

diameter p of agarose gels to their concentration C, in the form [18]

$$p = 140.7C^{-0.7}$$

At that time, there was a great confusion regarding this dependence, and all sorts of relationships were proposed, with factors $C^{-0.3}$, $C^{-0.5}$ and even C^{-1}. As it turned out, we were fairly close to the true dependence, established to be $C^{-0.75}$ [27], as also proposed by Slater *et al.* [28]. We then set out to study the properties of highly cross-linked gels and established the fact that one cross-linker, diallyltartardiamide, was in all practical effects an inhibitor of gel polymerization, thus ending a curious myth in biochemical analysis [19].

In 1981, we set out to reinvestigate gel polymerization kinetics as a function of temperature [15], amount and type of cross-linker [16] and type of catalyst [17]. Some basic rules were defined in those studies, such as the fact that the polymerization temperature strongly affects the quality of the final gel: polymerization at low temperatures (as recommended by several workers) resulted in poor conversion of the monomers into the polymer and in a modest incorporation of the cross-linker (Bis), which seems to form poorly reacting clusters at 5–10°C. Polymerization at 50°C was advocated and in fact adopted for achieving proper conversion of the Immobiline chemicals in the IPG technique [29]. However, it was only in 1984 that we first proposed a new reacting couple: acryloylmorpholine as a monomer and bisacrylylpiperazine as a cross-linker [20]; the idea for adopting these new monomers was based on the observation that they are amphiphilic, so that, once incorporated in a gel, they could favour reswelling also in aqueous–organic solvents. Later, we also adopted Trisacryl as a highly hydrophilic monomer [30], as simultaneously advocated by Kozulic *et al.* [31] for DNA analysis. For the reasons given in the Introduction, it is now clear that we were far from having reached a satisfactory solution. Gels based on the acryloylmorpholine monomer turned out to be too hydrophobic and to be opaque. Conversely, gels formed with Trisacryl were extremely hydrophilic but extremely susceptible to hydrolytic degradation even in mildly alkaline solutions (see Figs. 2 and 3). A novel, unique monomer seemed to be dimethylacrylamide: as shown in Fig. 2, it is fairly resistant to hydrolysis as a free species and extremely resistant in the polymer phase (Fig. 3A and B). We had in fact already adopted such a matrix, in a commercial preparation called Hydrolink, which however could only be used to some extent for DNA separations [32]. All our attempts at using it for protein separations were unsuccessful, as the monomer is already too hydrophobic ($P = 0.5$, see Table I). Hence the search for novel monomers has to continue; we have fund the rules that govern strong hydrolytic stability, but we still have to couple it to strong hydrophilicity, an essential feature for proper electrophoresis of proteins. Concerning the chemistry of cross-linkers, clearly DATD should be discarded, as discussed above. Also BAC should be used with great caution; given its extreme hydrophobicity, its use in protein separations should be discouraged. In fact, when first advocated, BAC was adopted only for nucleic acid analysis, as such polymers are not prone to hydrophobic interactions with the matrix.

Photopolymerization

Photopolymerization chemistry has now been well elucidated, and it appears to offer some unique features that could render it more preferable in biochemical applications than chemical polymerization: this process proceeds with a very high polymerization rate; by optimizing the reaction parameters very high conversions of monomer into the polymer phase can be obtained; photopolymerization does not need high-temperature conditions and proceeds successfully at room temperature; (d) photosensitizers, which can be used in very low concentrations compared with chemical initiators, do not oxidize proteins in gel matrices; and by varying the incident light intensity it is possible to produce gels of graded porosities. A unique finding appears to be the inverse proportionality between the elastic modulus and the gel pore size, as illustrated by the two curves in Fig. 6. As is known [26], the pore size of the polyacrylamide gels depends in a

non-monotonic fashion on the cross-linker concentration. The lower curve in Fig. 6 is the pore size evaluation performed by electrophoresis of protein markers for gel matrices polymerized at different cross-linker concentrations. The upper curve displays the dependence of the elastic modulus of polyacrylamide gels on the cross-linker concentration. As one can appreciate, in the latter instance a non-monotonic dependence has been obtained, with a maximum at 5%C, corresponding to the mobility (*i.e.*, porosity) minimum at the same concentration of cross-linker. Hence an increase in cross-linker concentration leads to an increase in elastic modulus and to a decrease in pore size at low concentrations of the cross-linker and to opposite dependences at concentrations higher than 5%, which illustrates the inverse proportionality of elastic modulus and pore size. Note that the change in the dependence of the elastic modulus on the cross-linker concentration is found to be connected with the transition from transparent to opaque gels. Thus, curiously, a "good elastic body" appears to be also a "body" exhibiting minimum permeability, both properties being coincident at 5%C (see also Fig. 5). A possible explanation could be that, at 5%C, the distribution of cross-links along the chains is fairly uniform, resulting in chain segments (or springs) possessing a good elastic memory. At high %C values, chain clustering or bundling could result in a loss of elastic memory and an inability to respond to a compression stress.

Laterally aggregated gels

How can the pore size of polyacrylamide matrices be manipulated? According to the Ogston model [33],

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

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.*, $r = 0$, here r is the fibre radius). In our case, given the substantial thickness of the gel fibres, their radius has to be subtracted from the computed value of the most frequent pore size population [34]. Thus,

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

There 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 manipulation of pore sizes in polyacrylamides by maintaining %T constant and progressively increasing %C seems to rely on the simultaneous shortening of L and thickening of the fibre diameter. Thus Fawcett and Morris [26] suggest that, while a 5%T gel has an average fibre diameter of 0.5 nm, a high %C gel has an upper value of fibre diameter of 6 nm. The procedure adopted by us is based on a different strategy: we drastically diminish the number of fibres per unit gel volume, while possibly not reducing the average L value (as %C is low and constant). This is accomplished by gelling the monomers in the presence of a hydrophilic polymer; we believe that such a polymer (notably PEG 10 000 or 20 000) acts by sequestering the water to the growing chains and forcing them to form large bundles, held together by preferentially (but perhaps not solely) interchain hydrogen bonds. It is in fact known that PEG coordinates large amounts of water around its coil (on the average, 1.5 water molecules per oxygen atom in the chain); this perturbation of the solvent phase could force the growing polyacrylamide strings to seek hydrogen bonding among themselves, rather than with the surrounding solvent. Once such large chain aggregates are formed, they are then stabilized in an irreversible structure by the crosslinks.

Fig. 9 gives a hypothetical representation of this "bundling" phenomenon. How large could such bundles be? We believe that the thickness of such fibres is much more than the 6 nm estimated by Fawcett and Morris [26]; a rough estimate of the diameters of these fibres, as clearly visible in the electron micrographs in Fig. 10D, suggests values of several hundred nanometres, in agreement also with the strong

light-scattering properties of such "laterally aggregated" gels. The process of drastically reducing n (the number of individual fibres per unit volume) has to have as a consequence enlargement of the average pore diameters; again, by measurements of pore sizes in Fig. 10D, we obtain an average value of 0.5 μm. We believe these values to be real, not artefacts of the sample manipulation prior to microscopic observations. The important step here was to equilibrate all samples in 2.5% PEG 10 000 prior to freezing and lyophilizing; in the absence of cryoprotectant, the gel membranes "exploded" and cavities as large as 100 μm could be seen. As a final remark, we emphasize that Gersten *et al.* [35] have also reported polymerization of polyacrylamide gels in presence of various hydrophilic polymers. Curiously, however, they did not report the huge increase in pore size that we experienced; on the contrary, they seemed to observe a general retardation of migration of SDS–protein complexes. Therefore, in the absence of additional data, it is impossible to compare our data with those of Gersten *et al.* [35].

ACKNOWLEDGEMENTS

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