Journal of Chromatography, 608 (1992) 343–348
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 194

Investigation of the properties of acrylamide bifunctional monomers (cross-linkers) by capillary zone electrophoresis

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ABSTRACT

A series of cross-linkers [N,N′-methylenebisacrylamide (Bis), N,N′-(1,2-dihydroxyethylene)bisacrylamide (DHEBA), N,N′-diallyltartardiamide (DATD) and N,N′-bisacrylylcystamine (BAC)] were investigated as potential candidates of a novel class of polyacrylamide matrices, exhibiting high hydrophilicity, high resistance to hydrolysis and larger pore sizes than conventional polyacrylamide gels. The most hydrophilic cross-linker (DATD) exhibited first-order degradation kinetics in 0.1 *M* NaOH, suggesting that such a structure is electronically unstable, whereas the other cross-linkers displayed first-order kinetics. In addition, when used in highly cross-linked gels (for increasing pore size), DATD acts as an inhibitor of gel polymerization. Another interesting finding is an inverse relationship between the partition coefficient of such cross-linkers and the incorporation efficiency; the more hydrophobic members of the family exhibit a reduced conversion from monomer into polymer, which is more pronounced in highly cross-linked gels. When measuring the partition coefficient, DATD and DHEBA appear to be highly hydrophilic whereas BAC, owing to the two sulphur atoms in the molecule, appears to be extremely hydrophobic. The use of BAC in gels for protein separations should therefore be avoided.

INTRODUCTION

In the accompanying paper [1], we reported the investigation of a series of monofunctional acrylamide monomers (in general mono- and disubstituted acrylamides), aimed at arriving at novel gel formulations. To this end, we utilized capillary zone electrophoresis (CZE) for studying such parameters as incorporation efficiency, hydrophilicity and resistance to hydrolysis. In this work, we investigated the same parameters for a series of cross-linkers, *i.e.*, the bi-functional agents used for producing the gel network. In addition to the standard molecule [N,N′-methylenebisacrylamide (Bis)], a host of them have been described over the years, and there are really no guidelines to suggest any strategy for their use. In 1976 O'Connell and Brady [2] reported the use of N,N′-(1,2-dihydroxyethylene)bisacrylamide (DHEBA) for casting reversible gels, as the 1,2-diol structure of DHEBA renders them susceptible to cleavage by oxidation with periodic acid. The same principle should also apply to N,N′-diallyltartardiamide (DATD) gels, as described by Anker [3]. Alternatively, ethylene diacrylate gels could be used, as this cross-linker contains ester bonds which undergo base-catalysed hydrolytic cleavage [4]. Another series of poly(ethylene glycol) diacrylate cross-linkers, as reported by Righetti *et al.* [5], also belong to the same category of ester derivatives. One of the latest addition to the series, N,N′-bisacrylylcystamine (BAC), contains a disulphide bridge cleavable by thiols and as such offers gel matrices which can be liquefied under mild and almost physiological conditions [6]. BAC-cross-linked gels were first proposed for the fractionation of RNA and they can be liquefied in an excess of β-mercaptoethanol or with dithiothreitol. As a novel class of cross-linkers, bisacrylylpiperazine has been reported [7] as a moiety able to copolymerize with good efficiency

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0021-9673/92/$05.00

with a modified monomer, N-acryloylmorpholine. Such a cross-linker was later reported to impart unique properties to gels to be silver stained, as it produced a perfectly clear background [8].

In this work, we selected the four most common cross-linkers (Bis, DHEBA, DATD and BAC) and studied their properties by CZE.

EXPERIMENTAL

Materials

The formulae of the four cross-linkers studied are given in Table I. Gels were prepared by using acrylamide as a monofunctional monomer and each of the four cross-linkers. Acrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED), the four cross-linkers and ammonium peroxodisulphate were obtained from Bio-Rad Labs. (Richmond, CA, USA). Mandelic acid and pK 9.3 Immobiline, used as internal standards in CZE runs, were purchased from Aldrich (Steinheim, Germany) and Pharmacia–LKB (Uppsala, Sweden), respectively.

Alkaline hydrolysis

All cross-linkers were dissolved (20 mM each) in 0.1 M NaOH and incubated at 70°C for up to 6 h. At 30-min intervals aliquots were collected and diluted in 0.1 M sodium borate buffer (pH 9.0) to 2.5 mM. After adding mandelic acid (2.50 mM) as an internal standard, the samples were analysed by CZE.

Incorporation efficiency

In order to check for the extent of conversion of the various monomers into the polymer, 5 ml of gel (made with the different cross-linkers in Table I) were polymerized in a test-tube under anaerobic conditions (protected by a thin film of water-saturated n-butanol). After polymerization (1 h, 50°C), the gel was extruded and homogenized and an equal amount of water was added (extraction was also performed in methanol). After stirring for 30 min, the gel phase was filtered through a Buchner funnel, the supernatant was diluted, internal standard (2.5 mM pK 9.3 Immobiline) was added and the mixture was filtered through an Ultrafree-MC Millipore filter (0.22 μm porosity) and analysed by CZE. No attempts were made to separate acrylamide from the cross-linkers, so that the incorporation efficiency refers to the sum of the two monomers.

Capillary zone electrophoresis

CZE was performed in a Beckman (Palo Alto, CA, USA) P/ACE System 2000 instrument equipped with a 50 cm × 75 μm I.D. capillary. Runs were performed at 25°C in a thermostated environment in 0.1 M borate buffer (pH 9.0). In all instances the migration direction was toward the negative electrode, which means that the acidic species (mandelic acid) are transported there by electroosmosis as they migrate electrophoretically toward the positive electrode. The samples were injected into the capillary by pressure (55 kPa), usually for 10 s. The calibration graph for each acylamido derivative analysed was constructed with the Beckman integration system Gold, with concentration points of 0.25, 0.50, 1.00, 1.25, 2.00, 2.50 and 3.50 mM. In each run either mandelic acid or the pK 9.3 Immobiline (2.50 mM) was used as an internal standard. Runs were usually

TABLE I

BIFUNCTIONAL ACRYLAMIDO DERIVATIVES

Formula	Name	M_r
$CH_2=CHCONHCH_2NHCOCH=CH_2$	N,N′-Methylenebisacrylamide (Bis)	154
$CH_2=CHCONHCHCHNHCOCH=CH_2$ OHOH	N,N′-(1,2-Dihydroxyethylene)bisacrylamide (DHEBA)	200
$CH_2=CHCH_2NHCOCHCHCONHCH_2CH=CH_2$ OHOH	N,N′-Diallyltartardiamide (DATD)	228
$(CH_2=CHCONHCH_2CH_2S-)_2$	N,N′-Bisacrylylcystamine (BAC)	260

performed at 15 kV and 50 μA with the detector set at 254 nm.

Partition coefficient

In order to establish a hydrophobicity scale, the various cross-linkers were subjected to partitioning in water–1-octanol as described by Purcell *et al.* [9]. The partition coefficient P is defined as the ratio between the molarity of a given compound in the organic phase and that in the aqueous phase. Partitioning was performed as follows: each monomer was dissolved (2 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 1800 g. All operations were performed at 25°C. The clarified solution was diluted, internal standard (2.5 mM pK 9.3 Immobiline) was added and the mixture was analysed by CZE as described above.

RESULTS

In order to check the stability of the four cross-linkers, they were subjected to hydrolysis in 0.1 M NaOH at 70°C for various lengths of time and then analysed by CZE for separation and precise determination of the degradation products. Fig. 1 shows a representative run of the CZE analysis of DHEBA, before and after hydrolysis (mandelic acid was added as an internal standard in all runs). A summary of all the hydrolysis data is given in Fig. 2. It can be seen that, whereas all the cross-linkers exhibit first-order degradation kinetics, as expected, one of them, DATD, displays a zero-order degradation process, suggesting that this structure is intrinsically unstable. This is noteworthy, as this cross-linker has recently been proposed in highly cross-linked gels for DNA analysis [10].

Table II summarizes the data obtained on the incorporation efficiency (of the two monomers) in a polyacrylamide gel subjected to standard polymerization conditions (1 h, 50°C) as routinely adopted in the field of electrophoresis [11]. Two phenomena are apparent: (a) in general, as the amount of cross-linker increases from 4 to 10% (or 30% in some instances), the incorporation efficiency diminishes: this is true for all the cross-linkers; (b) two of the cross-linkers (Bis and DHEBA) show very high incorporation efficiencies (> 90%) whereas the other two show progressively lower incorporation rates. A case in point is DATD, which, at progressively higher percentages of cross-linker, shows dramatic decreases in corporation efficiencies, down to as low as 50%.

Fig. 3 shows the hydrophobicity scale of the four cross-linkers, as obtained by partitioning in water–1-octanol phases. It is seen that DATD and DHEBA are highly hydrophilic, as expected given their vicinal diol structures, whereas BAC appears to be extremely hydrophobic, owing to the presence of an –S–S– bridge. These findings have important implications on the use of such gels for protein analysis, as discussed below.

DISCUSSION

The present findings suggest a strategy for the use of such cross-linkers, as outlined below.

Copolymerization efficiency

It appears that two main factors affect the copolymerization of cross-linkers with the standard monofunctional monomer (acrylamide): (a) the total percentage of cross-linker in the mixture (expressed as %C) and (b) the relative hydrophobicity of such compounds. Independently from the type of cross-linker, at progressively higher %C the incorporation efficiency diminishes (for both, the mono- and bifunctional monomers). This can be interpreted by assuming that, as %C progressively increases, the gel becomes more "knotty", *i.e.*, the chains become shorter and thicker. Given this gradual structural change, the monomers would have difficulty in diffusing in contact with the fibres and in finding the propagation point for the chain growth [12,13]. We have also seen in the accompanying paper [1] that, as monomers become more hydrophobic, their incorporation efficiency progressively diminishes. In fact, a unique inverse correlation has been found between the partition coefficient and rate of transfer into the growing polymer (see Fig. 7 in ref. 1). Thus, it is not surprising that BAC should lower the incorporation efficiency from >90% to only 77%. It might be asked then why DATD, being so strongly hydrophilic, exhibits even poorer chain transfer. This is in fact due to complete-

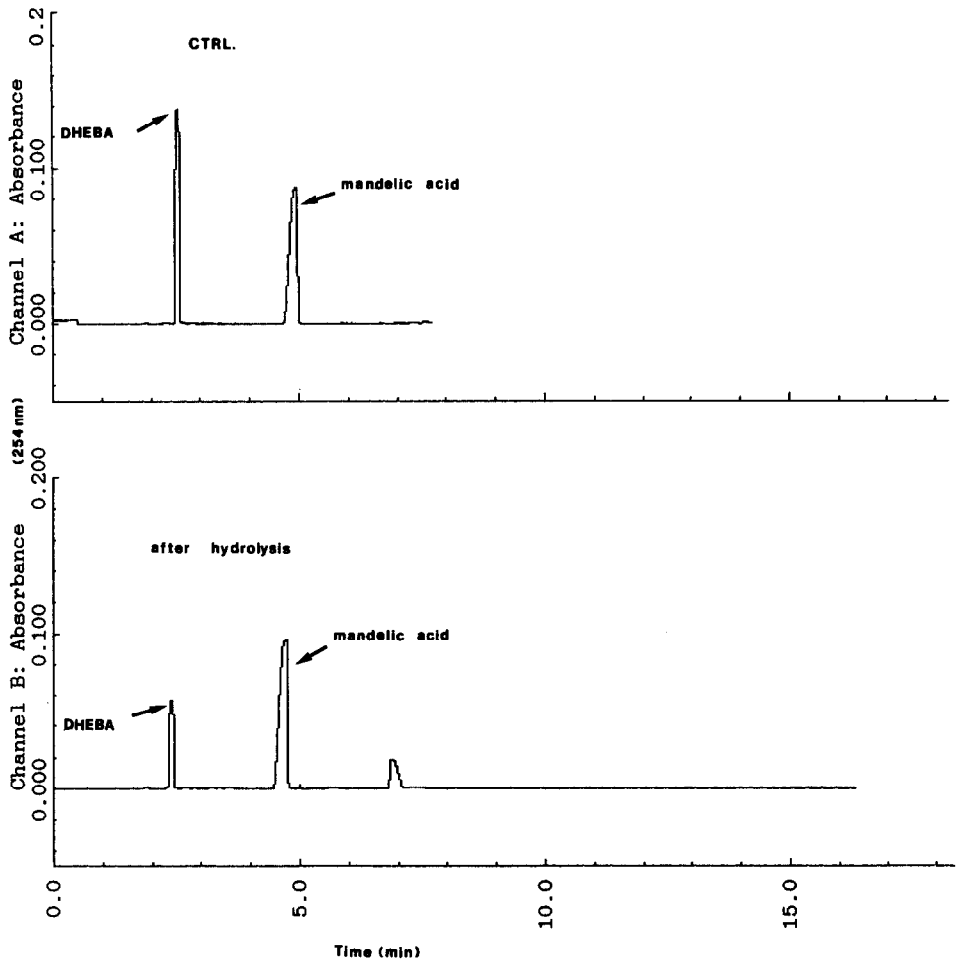

Fig. 1. Representative CZE runs for monitoring alkaline hydrolysis of cross-linkers. Conditions: 100 m*M* borate–NaOH buffer (pH 9.0), 15 kV, 86 μA at 25°C. Uncoated fused-silica capillary (50 cm × 75 μm I.D.). Beckman P/ACE 2000 instrument, monitoring at 254 nm. DHEBA = N,N′-(1,2-dihydroxyethylene)bisacrylamide; CTRL = control, before hydrolysis. In this and all subsequents runs the migration is towards the cathode.

ly different reasons: allyl derivatives are in general poorly reacting species. It has been reported that the C_m value (transfer constant to monomer) for most allyl compounds is usually 1000 times higher than for acrylamide [14]. Moreover, the r_i value (monomer reactivity ratio), which expresses the extent to which a monomer either propagates a chain by reacting with itself or by reacting with another, different monomer present in the mixture, is, for allyl derivatives, usually 100 times smaller than for acrylamide and, in some instances, is actually zero [15]. Thus, for all practical purposes, DATD, when copolymerized with acrylic double bonds, acts like an inhibitor of gel polymerization.

Use of different cross-linkers

It is now apparent why the "old pair" acrylamide–Bis has survived unscathed all these years: as a general rule, if we follow the old adage "similar dissolves similar", we can extrapolate it to copolymerization chemistry: "similar reacts with similar". The rule for selecting the appropriate copolymerizing pair is that they should be very similar molecules, like acrylamide and Bis. Thus, in 1984,

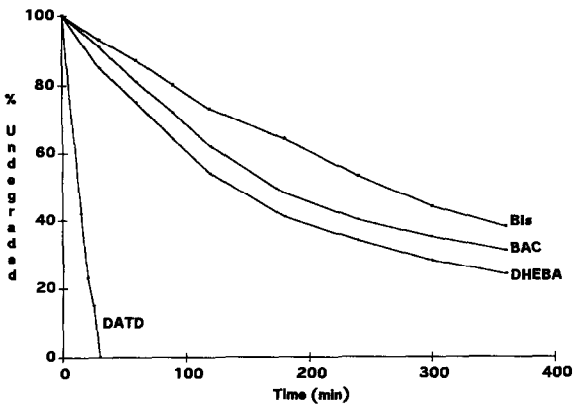

Fig. 2. Kinetics of hydrolysis of different cross-linkers. Hydrolysis was performed in 0.1 *M* NaOH at 70°C for the times indicated. The amounts were assessed by collecting in triplicate at each point, neutralizing and injecting into the CZE instrument (Beckman P/ACE 2000). Conditions: 100 m*M* borate–NaOH buffer (pH 9.0), 15 kV, 86 μA at 25°C. Uncoated fused-silica capillary (50 cm × 75 μm I.D.). Peak integration with the Beckman system Gold (mandelic acid was used as an internal standard). Abbreviations as in Table I. Note that whereas all the other cross-linkers exhibit first-order kinetics, DATD follows zero-order degradation kinetics.

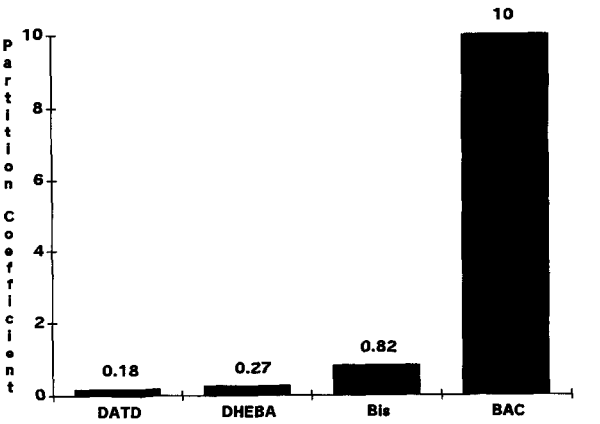

Fig. 3. Hydrophobicity scale for the four cross-linkers. It was obtained by partitioning in water–1-octanol at room temperature and determining the concentrations in the two phases by CZE. Conditions: 100 m*M* borate–NaOH buffer (pH 9.0), 15 kV, 86 μA at 25°C. Fused-silica capillary (50 cm × 75 μm I.D.). Peak integration with the Beckman system Gold (mandelic acid was used as an internal standard). Abbreviations as in Table I.

when we reported the novel monomer N-acryloylmorpholine [7], we synthesized as a co-reacting species bisacrylylpiperizine; this pair produced excellent gels for electrophoresis. It does not seem a promising proposition to try to copolymerize allylic with acrylic moieties, as suggested recently [10]; in all practical effects, the former acts like a radical sink, inhibiting the gel formation. The notion of using an "inhibitor" for polymerizing a gel does not seem to be based on sound polymer chemistry. In addition, DATD is electronically unstable, as it displays zero-order degradation kinetics (probably by the same N,O-acyl transfer mechanism reported for trisacryl) [16]. Given such severe drawbacks, the use of DATD as a cross-linker should in general be avoided. A word of caution should also be expressed about the use of BAC: its relative hydrophobicity seems to inhibit to some extent the conversion of monomers into the polymer. Moreover, if used for protein separations, as suggested elsewhere [17], there could be another major risk: because, for the purpose of liquefying the gel for sample recovery, highly cross-linked gels should be used, this would greatly increase the matrix hydrophobicity, with a concomitant risk of hydrophobic interaction of the protein sample with the matrix. This would not, of course, discourage the use of BAC-cross-linked gels for nucleic acid analysis, as such polymers are not prone to hydrophobic interaction with the matrix.

TABLE II

INCORPORATION EFFICIENCY AS A FUNCTION OF PERCENTAGE OF CROSS-LINKER (%C)

All experiments refer to standard polymerization conditions: 1 h at 50°C.

Monomer[a]	%C	Incorporation (%)[b]
BAC	4	77
	10	72
Bis	4	95
	10	90
DHEBA	4	90
	10	85
DATD	4	65
	10	58
	30	48

[a] %C = g Bis/%T; T = g acrylamide + g Bis per 100 ml of solution; all gels had variable %C at constant %T (total monomers, 6%T).

[b] The incorporation efficiency refers to the sum of the two monomers (acrylamide + cross-linker).

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Agenzia Spaziale Italiana (ASI) and the European Space Agency (ESA) for gel polymerization in microgravity and by the Consiglio Nazionale delle Ricerche, Progetti Finalizzati Chimica Fine II and Biotecnologie e Biostrumentazione.

REFERENCES

1 C. Gelfi, P. de Besi, A. Alloni and P. G. Righetti, *J. Chromatogr.*, 608 (1992) 333.
2 P. B. H. O'Connell and C. J. Brady, *Anal. Biochem.*, 76 (1976) 63–76.
3 H. S. Anker, *FEBS Lett.*, 7 (1970) 293–296.
4 P. N. Paus, *Anal. Biochem.*, 42 (1971) 327–376.
5 P. G. Righetti, B. C. W. Brost and R. S. Snyder, *J. Biochem. Biophys. Methods*, 4 (1981) 347–363.
6 J. N. Hansen, B. H. Pheiffer and J. A. Boehnert, *Anal. Biochem.*, 105 (1980) 192–201.
7 G. Artoni, E. Gianazza, M. Zanoni, C. Gelfi, M. C. Tanzi, C. Barozzi, P. Ferruti and P. G. Righetti, *Anal. Biochem.*, 137 (1984) 420–428.
8 D. Hochstrasser and C. R. Merril, *Appl. Theor. Electron.*, 1 (1988) 35–40.
9 W. P. Purcell, G. E. Bass and J. M. Clayton (Editors), *Strategy of Drug Design: a Guide to Biological Activity*, Wiley-Interscience, New York, 1973, pp. 126–143.
10 L. Orbán and A. Chrambach, *Electrophoresis*, 12 (1991) 241–246.
11 B. Bjellqvist, K. Ek, P. G. Righetti, E. Gianazza, A. Görg, R. Westermeier and W. Postel, *J. Biochem. Biophys. Methods*, 6 (1982) 317–339.
12 A. Bianchi-Bosisio, C. Loeherlein, R. S. Snyder and P. G. Righetti, *J. Chromatogr.*, 189 (1980) 317–330.
13 P. G. Righetti, in R. C. Allen and P. Arnaud (Editors), *Electrophoresis '81*, Walter de Gruyter, Berlin, 1981, pp. 3–16.
14 L. J. Young, G. Brandrup and J. Brandrup, in J. Brandrup and E. H. Immergut (Editor), *Polymer Handbook*, Interscience, New York, 1966, pp. II-77–II-79.
15 H. Mark, B. Immergut and E. H. Immergut, in J. Brandrup and E. H. Immergut (Editors), *Polymer Handbook*, Interscience, New York, 1966, pp. II-141–II-175.
16 A. P. Phillips and R. Baltzly, *J. Am. Chem. Soc.*, 69 (1947) 200–205.
17 R. Bartels and L. Bock, in C. Schafer-Nielsen (Editor), *Electrophoresis '88*, VCH, Weinheim, 1988, pp. 289–294.