# Observations on the structure of a polyacrylamide gel from electron micrographs

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We compare the results of electron microscopy from replicas obtained by the freeze-etch technique applied to a polyacrylamide gel and a polyacrylamide solution at the same concentration. The gel differs from the polymer solution in its preparation only by the presence of *N,N'*-methylene bisacrylamide which crosslinks the linear chains. The comparison is made on samples prepared in a 75% water–25% glycerol solution to minimize crystallite formation during the quenching step. The structure of the polymer solution appears to be much more homogeneous than that of the gel at the same magnification level. Analyses of the micrographs with the help of diffraction patterns indicate that the gel has a much broader distribution of 'pore' or 'mesh' sizes than the corresponding polymer solution. Some support for the suggestion of aggregation of crosslink molecules to form somewhat denser regions (heterogeneities) in the gel can be deduced from the micrographs obtained from gels prepared in pure water and in a water–glycerol mixture.

**(Keywords: structure; polyacrylamide gel; electron microscopy; crystallites; aggregation; crosslinking)**

## INTRODUCTION

The results from a variety of experiments such as swelling[1], permeation[2], mechanical measurements[3] and light scattering measurements[4,5] on polyacrylamide gels have either supported or have been interpreted in terms of the presence of heterogeneities in the network. The aim of this paper is to make the concept of 'heterogeneity' more concrete by visualizing the structure of the gel through the technique of freeze-etch electron microscopy. We also compare the structure of a gel prepared in a water–glycerol solution to that obtained under identical conditions for the uncrosslinked polymer system. Although the freeze-etch technique makes it possible to study gels and solutions without exposing them to the severe perturbations introduced by more conventional preparatory techniques, the delicate freezing procedure is faced with the major problem of the formation of ice crystals during quenching and the distortion or destruction of structure if the growth of the crystals is not prevented. The main parameter that governs the size of crystallites formed in the quenched sample is the ratio of the freezing rate to the rate of ice crystallization. It is impossible to completely achieve the vitreous state for freezing rates slower than a critical freezing rate, which is of the order of $10^6\,K\,s^{-1}$ for pure water and $10^4\,K\,s^{-1}$ in biologically active cells[6]. For dilute polyacrylamide gels prepared in water, the critical rate probably has an intermediate value. The experimental freezing rate is determined by the freezing method, the cooling medium, and the sample (its size, geometry and composition). It is therefore necessary to maximize the freezing rate by optimizing these factors. The composition of the sample can be modified by the addition of a cryoprotectant with the underlying assumption that the cryoprotectant does not affect the structure. For water-base specimens, glycerol is considered as the best cryoprotectant and although its effect on the formation of polyacrylamide chains and networks is not known, it is hoped that this effect does not play a role in the comparison of gels and polymer solutions formed under identical conditions. In the next section we describe our procedure for the preparation of the samples and the freeze-etch replicas. It is followed by the presentation of our results in the form of micrographs and our interpretation of these micrographs.

## PREPARATION OF FREEZE-ETCH REPLICAS

### *Sample preparation*

Polyacrylamide gels were prepared by an established redox reaction employing ammonium persulphate and tetra methyl-ethylene-diamine[2,7]. Acrylamide (5 g), *N,N'*-methylene bisacrylamide (0.133 g), ammonium persulphate (40 mg) and TEMED (0.16 ml) were dissolved in water with a total volume equal to 100 ml. The pH value of the mixtures were between 8.5 and 9.0, and no buffer solutions were introduced. Polyacrylamide solutions were prepared in the same way as above except that the crosslinking agent (*N,N'*-methylene bisacrylamide) was not introduced into the mixtures. Glycerinated samples were prepared following the above formulations and procedures except that a 25% (by volume) glycerine in water mixture replaced the pure water as solvent.

Filtrations of the solutions were performed using a 0.22 $\mu$m pore size membrane filter before the start of the reaction to eliminate dust and impurities which enhance heterogeneous ice crystal nucleation. Sealable capillary tubes with ID = 0.4 mm were used as gelation moulds. Before gelation, the solutions of mixtures were introduced into the capillary tubes by capillary suction. The capillary

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tubes were then sealed and stored in a room temperature of 25° ± 2°C.

*Freezing, fracturing and etching*

Balzer's Freeze-Etch Equipment consisting of a Balzer's Electron Beam Evaporation set-up (EVM052) with an Electron Beam Gun (EK552) was used to make the freeze-etched surfaces and the carbon–platinum replicas studied here. After curing at 25°C, the gels were removed from the capillary tubes by hydraulic pressure and loaded onto modified Balzer's gold cups which were designed to hold specimen of the minimum manageable size (0.4 mm diameter × 0.4 mm length) for faster cooling rates. A two step standard freezing technique was used to freeze the samples[8,9]. Gold cups loaded with gel samples were first frozen in liquid propane at its melting point (−190°C) and subsequently frozen and stored in a liquid nitrogen bath (−196°C). The direct freezing of samples in liquid nitrogen could cause film-boiling which leads to poor heat transfer rate. The liquid propane in coexistence with solid propane can maintain its temperature at its melting point during the entire sample freezing step and provides the fastest freezing rate known of all the available freezing methods[8].

Cleavage of the specimen was performed at −100°C to expose a clean surface to a vacuum environment ($P \leqslant 2 \times 10^{-6}$ Torr). During the etching step, a cold trap (precooled knife at the liquid nitrogen temperature of −196°C) was placed in Balzer's set-up near the sample to trap the subliming water vapour to serve as an anti-contamination device. The etching time was 5–10 s for nonglycerinated samples, and 1 min for glycerinated samples.

*Replication*

A platinum–carbon shadowing film was cast at a 45° angle on the etched surface of the specimen to show its structure. The electron beam gun (Balzer's EK552) was used to evaporate platinum and carbon, and a quartz crystal monitor was used to control the thickness of the deposited film. A pure carbon backing film was further deposited to increase the mechanical strength of the replica. Usually a 10–15 Å thick layer of heavy metal such as the platinum used here provides sufficient contrast and a 100–250 Å thick layer of carbon provides sufficient mechanical stability to the replica. The replicas were floated on the surface of distilled water or on glycerinated solution depending on the case and were transferred to a 5% sodium hyperchloride solution to remove organic impurities. After successive transfers in cleaning solutions and finally in distilled water, the replicas were picked up on formvar-film coated grids to furnish micrographs on a JEM-7A transmission electron microscope. The micrographs were taken at 80 or 100 KV accelerating voltage.

## RESULTS AND INTERPRETATION

During freezing, nucleation sites will grow to form ice micro-crystals as the first step of solidification; however, for fast freezing rates, most of the water and polymer mixture solidifies in the amorphous state to form a hypereutectic mixture before the true eutectic composition is reached. All the free unbound frozen water is sublimable during etching and leaves pits in the matrix to form a 3-dimensional structure. The formation of ice crystals would distort the original structure of the gels because as the crystals grow larger (if allowed by a poor sample preparation technique) they will draw water molecules from other regions and the final structure will be governed by ice formation[10]. Owing to the best available freezing method (liquid propane at its melting point used as the cooling medium with a freezing rate lying above 5600 K $s^{-1}$) and the minimum manageable sample size (0.4 mm diameter × 0.4 mm length) employed in our delicate sample preparation procedure, we trust that ice crystallization has been minimized to a degree that would not blur the interpretation of our results.

*Figure 1* shows the results for a polyacrylamide gel in water obtained by the preparation procedure discussed above. We can observe the controversial fibril and open cellular structure observed with other gels by other investigators[10–13]. However, the largest pore sizes we have obtained are of the order of 0.1 μm. These are much smaller than those reported by other groups[11,12]. The fibrils represent the polymer phase, and pores represent the free water phase. It is interesting to note that the fibrillar structure is not entirely random. As we can see where indicated by the arrow in this picture, there must be regions with much higher fibril concentration and finer pores than average in the system. The size of this particular highly crosslinked region is also of the order of 0.1 μm. This degree of heterogeneity agrees with the results of dynamic light scattering performed under a microscope by Weiss[5].

As with all hydrogels, however, polyacrylamide gel structures are very easily distorted by ice crystal formation during the sample preparation steps. The overall open cellular structure observed in *Figure 1* has some

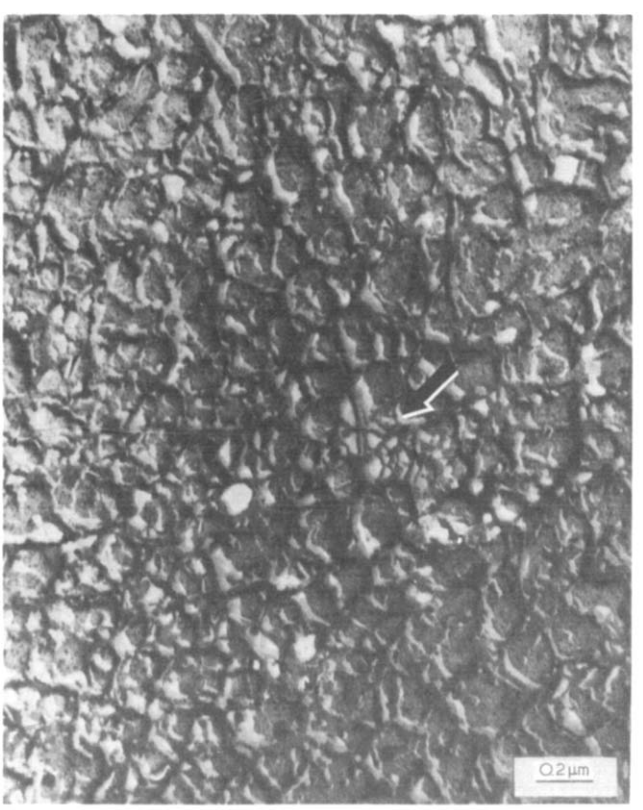


**Figure 1** Electron micrograph from a polyacrylamide gel composed of 5% polymer in water with a crosslink to monomer ratio of 2.66%. (Magnification 58 000×)

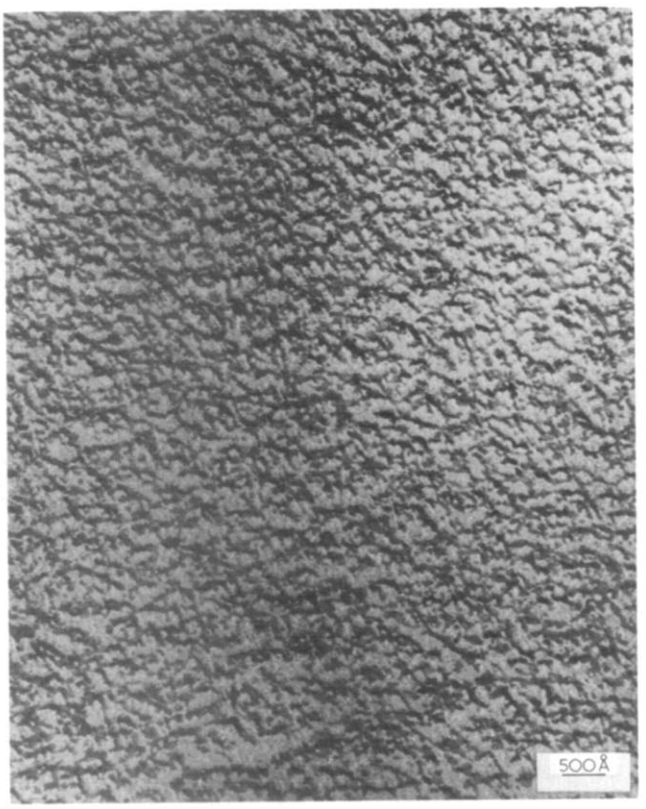


**Figure 2** Electron micrograph from a polyacrylamide gel composed of 5% polymer in a 25% glycerine in water solution and a crosslink to monomer ratio of 2.66%. (Magnification 200 000×)

similarity (on a much smaller scale) to the distorted structure due to ice crystallization[14] and may throw into question the validity of that structure. Since ice crystallization can be drastically reduced by the introduction of cryoprotectants such as glycerine, we examined the structure of glycerinated gels. Furthermore, to reduce the uncertainty caused by the addition of glycerine to our samples, we also studied the structure of glycerinated polyacrylamide solutions under the identical conditions of concentration and sample preparation as those used for the glycerinated gels.

The micrograph of a glycerinated gel is presented in *Figure 2*. The picture exhibits fine fibrils and a pore structure. The fibrils, with dimension of diameter of the order of 40 Å, are non-randomly distributed and seem to consist of a network of very fine dimensions with a wide distribution of pore sizes ranging from a few tens of angstroms to several hundreds of angstroms. Fine fibril network structures have also been observed in other gels by freeze-etching technique as well as by the thin section technique[10]. *Figure 3* displays the micrograph of a glycerinated polyacrylamide solution at the same concentration as the gel of *Figure 2*. Some isolated islands appear in the micrograph and the pore size distribution appears much narrower than in the previous case leading to a fairly homogeneous dispersed system.

We believe that the inherent differences between *Figures 2* and *3* are primarily due to the less homogeneous nature of the polyacrylamide gel structure. To achieve a more quantitative description of these differences we analysed the negatives of the micrographs presented in *Figures 2* and *3* by photodiffraction using a neon laser beam diffractometer. We used standard principles of photodiffraction[15] to interpret our diffraction patterns.

*Figure 4* gives the diffraction pattern from the micrograph of the glycerinated polyacrylamide solution at a magnification of 21 000. The dumbbell shape pattern obtained instead of a continuous diffuse 'ring' is due to the orientation introduced by the unidirectional shadowing effect of the platinum-carbon film. An additional diffraction pattern in the form of a cross at the central beam spot is caused by the rectangular aperture used here[15]. A continuous diffuse ring is commonly interpreted as a distribution of spacings between any two neighbouring pores in real space indicating a broad distribution of pore sizes in the polyacrylamide solution. A grid with a lattice spacing in real space of 6.04 lines $mm^{-1}$ and its diffraction pattern served as a calibrating standard for distances in both reciprocal space and in real space. The boundary of the continuous diffuse ring pattern in *Figure 4* corresponds to 90 Å in real space. From the diffraction pattern we may then conclude that the spacings between any two neighbouring pores are 90 Å or bigger with very few spacings below 90 Å. Indeed *Figure 3* shows that the spacings between two neighbouring pores lie between 90 Å and 400 Å.

*Figure 5* gives the diffraction pattern from the micrograph of the glycerinated polyacrylamide gel at a magnification of 24 000. It also shows a continuous diffuse ring, and the non-perfect ring pattern is again caused by the orientation introduced by the shadowing procedure. There are, however, several differences between this continuous diffuse ring and the result from polymer solutions on *Figure 4*. A strong intensity area at the centre of this picture forms a sort of 'inner' high intensity ring

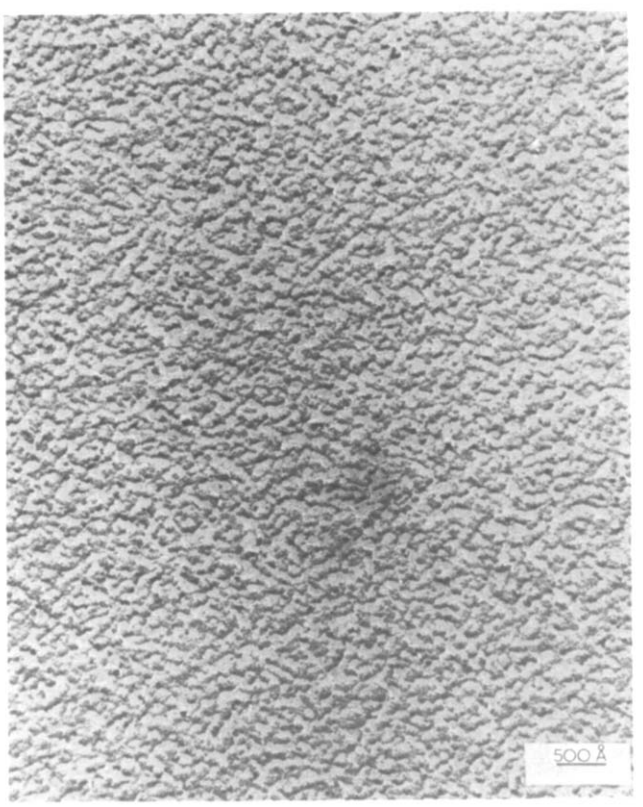


**Figure 3** Electron micrograph from a polyacrylamide solution composed of 5% polymer in a 25% glycerine in water solution. (Magnification 200 000×)

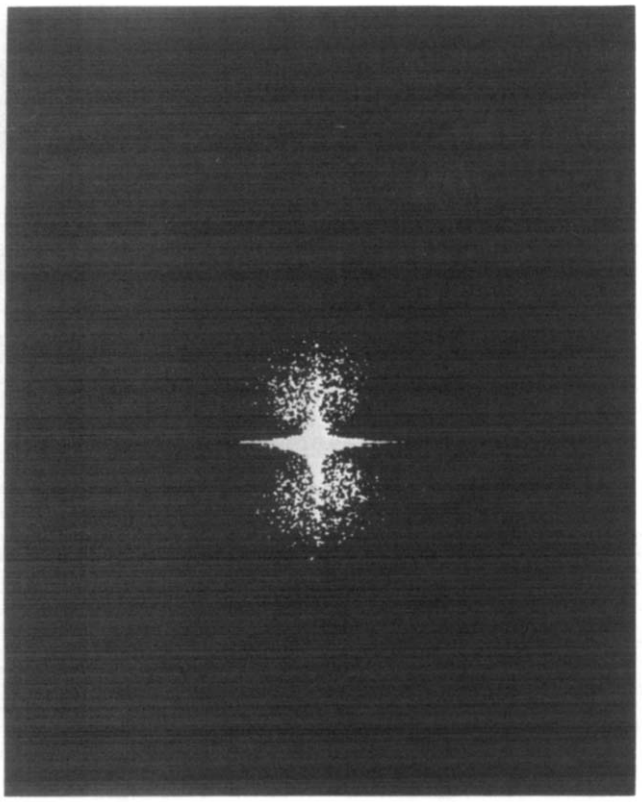

**Figure 4** Diffraction pattern of the micrograph from the glycerinated polymer solution at a magnification of 21 000

surrounded by an outer lighter diffuse region with an outer boundary corresponding to 60 Å in real space dimension. The boundary of the 'inner' ring corresponds to approximately 95 Å in real space distance. From this intensity distribution we may conclude that the pore size spacings in polyacrlyamide gel are primarily 95 Å or bigger with some pore sizes between 95 Å and 60 Å; pores with sizes under 60 Å must be very few. From *Figure 2* we can also deduce by visual inspection that the largest pore sizes must be of the order of 700–800 Å and that the smallest pore sizes tend to be clustered together (also evident in *Figure 1*). The non-uniformity of pore size distribution and the much wider range of sizes observed in the gel suggest that the structure of polyacrylamide gels is fairly inhomogeneous by comparison to the polyacrylamide solutions at the same concentration for the polymer concentration (5 g $dl^{-1}$) examined here. *For simplicity*, one can talk therefore about inhomogeneities in the gel that consist of regions of much narrower mesh sizes (or higher polymer density)[1,2].

## DISCUSSION AND CONCLUSION

Previously reported torsional pendulum measurements of the storage shear modulus show that the number of effective crosslinks linking various strands together in the gel network is only a very small percentage (5–10%) of the actual number of crosslink molecules introduced into the system[2,3,17]. These results suggest that the crosslink molecules must be segregated to some degree to form a sort of microstructure inside the network matrix as originally proposed by Richards and Temple[1]. The presence of these microstructures caused by non-random crosslinking and cyclizations appears to cause the main difference in structure between polyacrylamide gels and polyacrylamide solutions. The electron micrograph obtained on a gel prepared in water (*Figure 1*) shows clearly a network structure with some regions very highly crosslinked compared to the rest of the network. The size of these regions ('inhomogeneities') is estimated to be of the order of 0.1 $\mu$m from the micrograph. A previous attempt at quantifying the size of the 'inhomogeneities' in polyacrylamide gels was carried out by Weiss[5] using a microscope in a dynamic light scattering experiment monitoring the photon correlation functions from successively smaller scattering volumes. For large scattering volumes the correlations are identical; volumes below a length scale corresponding to 5000 Å led however to very different results for the gels although still giving identical results for an homogeneous solution of polystyrene latex spheres of 880 Å in diameter. These results suggest that the largest size of the 'inhomogeneities' lie between 0.088 $\mu$m and 0.5 $\mu$m which covers the range of the size of the highly crosslinked regions observed in our electron micrographs. From an analysis of the total intensity (static) light scattering measurements of a more dilute gel (3% polyacrylamide), Geissler *et al.*[4] conclude that the size of their inhomogeneities is of the order of 300 Å. They report, however, large fluctuations in total intensity at a fixed angle, and we have made similar observations on our gels[14]. Despite these fluctuations a definite trend towards higher intensities at lower angles is present in the gel but totally absent from the polymer solution of the same concentration. The total intensity of the latter is very stable and angular independent in the angle range of 60°–110° we have studied[14]. The angular dependence of the total scattered intensity in the gels can be interpreted in terms of the scattering due to small regions of higher polymer density (and therefore of

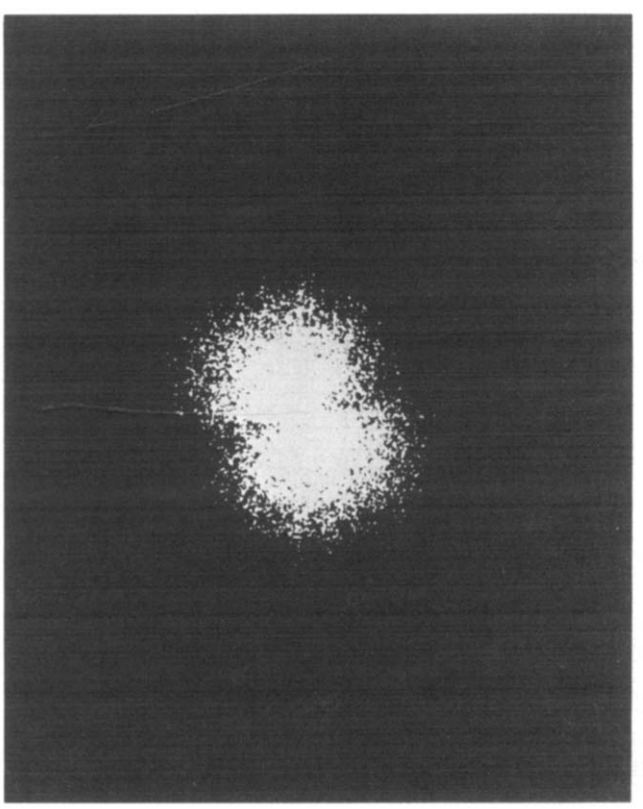

**Figure 5** Diffraction pattern of the micrograph from the glycerinated gel at a magnification of 24 000

different refractive index) than their surroundings; the fluctuations observed at a fixed angle would be then due to the motion in and out of the scattering volume of these regions. This interpretation would be in agreement with the observations we have made above from the electron microscopy of gels. Although the freeze-etch technique may be faced with difficulties in controlling the freezing step and with uncertainties regarding the interpretation of the micrographs obtained, the technique shows clear differences between polyacrylamide polymer solutions and gels at the same concentration prepared under identical conditions. The micrographs from the gel exhibit a much broader distribution of pore sizes and the presence of clusters of small pore sizes which may be taken in a crude way as representing the 'inhomogeneities' observed by light scattering and affecting the mechanical, thermodynamic and transport properties of the gels.

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