Effect of "Glutaraldehyde" Functionality on Network Formation in Poly(vinyl alcohol) Membranes

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ABSTRACT: The use of higher-functionality oligomers of glutaraldehyde on network formation was investigated and compared with glutaraldehyde monomer in step-growth reactions. The effect of using such oligomers in network formation depends on the stoichiometry, which alters either the branching or both the branching and crosslinking of the network. This was demonstrated in the properties of poly(vinyl alcohol) (PVA) networks crosslinked with glutaraldehyde using cryogenic scanning electron microscopy, water swelling studies, and protein transfer across membranes. General guidelines were given for the proper use of glutaraldehyde solutions. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 96: 780–792, 2005

Key words: hydrogels; functionalization of polymers; oligomers; stoichiometry; membranes

INTRODUCTION

Glutaraldehyde finds extensive application in the biological sciences as a fixative for electron microscopy, as a tanning agent for leather, as a crosslinking agent for chemical modification of proteins, and as a sterilizing agent.[1–3] In recent years, glutaraldehyde use has been widened to prepare crosslinked polymer gels for bioseparations, including electrophoresis networks, size-exclusion membranes, and also in the development of new drug release agents for biomedical purposes.[4–7]

It has been generally accepted that many commercial-grade "glutaraldehyde" solutions do not contain the dialdehyde alone.[1,2,8–11] Recent papers refer to publications dating back to the early 1960s that consistently describe the inherent problems faced in using commercial-grade glutaraldehyde and the lack of reproducibility has been recognized for a long time.[1,11–15] Of particular interest are aldol condensation products such as the oligomeric α,β-unsaturated aldehydes shown in Figure 1.[8,9,16] These molecules have a higher potential functionality than that of glutaraldehyde and this article will focus on a comparison of these oligomers and glutaraldehyde.

Although the problems with commercial-grade glutaraldehyde solutions are well known and it is recognized that high-integrity glutaraldehyde solutions are available, it has become common practice in the biological sciences to use technical-grade commercial glutaraldehyde "out of the bottle" without further consideration for purification or characterization.[3,6,7,11,17] Some studies claim these solutions to be more effective than distilled glutaraldehyde.[18–20] Others have loosely applied the term "glutaraldehyde" to describe solutions containing species with potential functionality of up to four (i.e, the mixture of glutaraldehyde with dimer and trimer oligomers).[21]

Polymer networks are formed using either chain-growth or step-growth reactions. When the amount of crosslinker is changed in either of these, the effects on the systems can be dramatically different. Excess of a divinyl crosslinker such as *N*,*N*′-methylene bisacrylamide (Bis) in a chain-growth reaction gives a tighter network. However, in step-growth systems stoichiometry can be critical and the amount of crosslinker is important.

The present work uses a functionality approach of "glutaraldehyde" solutions to examine network formation. It is widely perceived that the oligomers of glutaraldehyde, which have more than two aldehyde groups per molecule, would increase the functionality of the system as a whole. Herein it is shown that this is not necessarily correct. Factors including the effects of stoichiometry, interpretation of the analysis of commercial glutaraldehyde, and the functionality of the nonaldehydic component must also be taken into account when using commercial glutaraldehyde solu-

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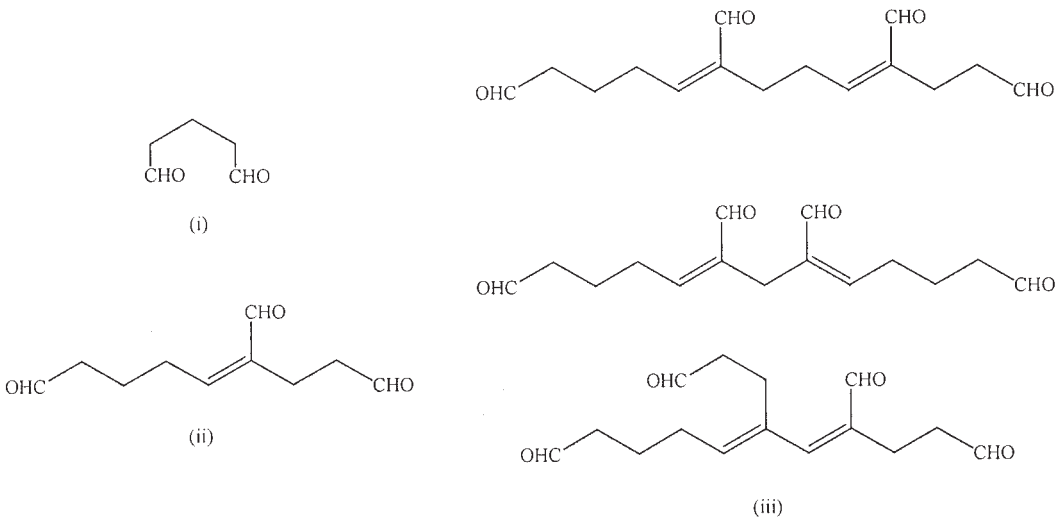

Figure 1 Structure of (i) glutaraldehyde, and aldol condensation products (ii) dimer and (iii) possible trimers.

tions. This is illustrated by reference to the preparation of crosslinked poly(vinyl alcohol) (PVA) membrane applications—ultimately of interest for biological separations. However, the results have general application.

EXPERIMENTAL

Materials

Glutaraldehyde [25% (w/v) solution] was obtained from Asia Pacific Specialty Chemicals Ltd. (Victoria, Australia). Poly(vinyl alcohol) (PVA; Mw 27,000, 98.5% hydrolyzed) was generously supplied by Clariant (Frankfurt, Germany). All other reagents were of analytical grade and obtained from Sigma Aldrich Chemical Company (Sydney, NSW, Australia) and purified using conventional methods.

Instrumentation

A Philips XL 30 field emission scanning electron microscope (SEM) instrument (Philips Inc., Melville, NY), equipped with an Oxford CT 1500 HF chromotransfer system, was used to take images of the PAAm gels at 2 keV.

A Gradiflow BF200™ unit was used for preparative electrophoresis and for gel electrophoresis a Gradipore Model 250-4 power supply, Mini vertical gel system, and a Micrograd vertical electrophoresis unit were used (Gradipore Ltd., Sydney, NSW, Australia). A Cary 3E UV–visible spectrophotometer (UV–vis; Varian Inc., Palo Alto, CA) was used to examine absorbance of protein and glutaraldehyde solutions.

Mass spectrometry was recorded at 20 V with a Quattro II (Micromass, Ontario, Canada) electrospray mass spectrometer (ES/MS). Data along with intensity (% of base peak) and the molecular ions ($M^+ \cdot$ or $MH^+ \cdot$) are reported.

Isolation and characterization of "glutaraldehyde" from commercial solution

A commercial-grade "glutaraldehyde" solution was stirred with activated charcoal, filtered through basic alumina filter aid, and saturated with NaCl.[22] This solution was extracted using diethyl ether and concentrated *in vacuo.* The crude "glutaraldehyde" was distilled under vacuum (b.p. 45°C, 3–5 mmHg, 12.5% yield, n_D^{25} 1.4339) and diluted to make a 25% (w/v) solution of glutaraldehyde with MilliQ water and purged with Ar_g and stored at 4°C (lit. b.p. 187–189°C, 760 mmHg, decomp., 71°C, 10 mmHg, pure glutaraldehyde n_D^{25} 1.4338).[23]

Determination of aldehyde content

The number of aldehyde groups in "glutaraldehyde" solutions was determined by reaction with sodium sulfite to form a bisulfite addition product and a stoichiometric equivalent of base.[24] The base was titrated with standardized HCl solution (0.0506*M*), using thymolphthalein indicator to observe the endpoint of the reaction.

On a weight basis, the dimer of glutaraldehyde has been treated as equivalent to 2 moles of glutaraldehyde. A molecule of water is eliminated in forming the dimer but this water remains in the reagent. This means that an aliquot containing 2 moles of pure glutaraldehyde is equivalent by weight to the same aliquot containing 1 mole of the dimer or an equal aliquot with 2/3 mole of the trimer and so on. Subsequently herein the term "out of the bottle" will be used

to describe the commercial technical grade glutaraldehyde where acidic components have been removed from the reagent.

Preparation of poly(vinyl alcohol)–"glutaraldehyde" gels

A poly(vinyl alcohol) (PVA) solution was prepared by dissolving the appropriate mass of PVA in distilled water (75–80 mL) at 85°C. This was cooled to room temperature and an acid catalyst, HCl (3.33 mL 6.0*M* solution), was added and diluted with distilled water to 100 mL. This gave a final solution of PVA at the required concentration (% w/v) and 0.2*M* HCl. This solution (10 mL) was mixed with the "glutaraldehyde" solution (25% w/v) and charged to a container purged with $Ar_{(g)}$.

Scanning electron microscopy (SEM)

Gel morphology was examined using cryogenic SEM to prevent collapse or alteration of the structure of the gel network on drying. Gels (5 × 5 mm) were mounted vertically on a SEM stub with a nonconductive glue and frozen at −198°C in liquid nitrogen. The top was fractured off and the gel then warmed to −85°C for 90 min while subliming water from the gel under reduced pressure. The sample was again cooled to −198°C and images of the fractured gel were taken at various magnifications.

Water swelling

Water absorbed by the PVA gels was observed. Gels (4 × 2.5 × 0.5 cm) were cut, weighed, and dried at 30°C for 24 hrs, *in vacuo.* Dried gels were weighed and put into 50 mL distilled water at 20°C. These were removed at regular intervals, patted with filter paper to remove excess surface water, weighed, and reimmersed. The dried gels were weighed and the water-swelling ratio was determined for each gel as the mass (g) of water absorbed divided by the dry gel mass (g).

Membrane preparation and preparative electrophoresis

Membranes were prepared and separation cartridges assembled as described previously.[25] Preparative electrophoresis was used to assess membranes for pore size difference. For all tests 40 m*M* Tris–glycine buffer was used as described previously.[25]

Protein transfer

Preliminary screening of membranes was performed using a variety of proteins. Protein transfer was initially examined qualitatively using the prepared membranes and Tris–glycine buffer system. Protein samples were prepared using 15 mL of bovine fibrinogen (340 kDa, 1 mg mL^{-1}), a large glycoprotein, or a smaller protein such as bovine serum albumin (BSA, 67 kDa, 2 mg mL^{-1}). Protein solution was placed in stream 1 of the Gradiflow unit, whereas stream 2 was filled with buffer.[25]

Visualization of protein transfer

Gradient gel electrophoresis was performed using Gradipore Ltd. gradient polyacrylamide (PAAm) gels (product code: ng21-420) to facilitate visualization of protein transfer after preparative electrophoresis. For gel electrophoresis, fractions (50 μL) were taken from both preparative electrophoresis reservoirs at 30-min intervals. Fibrinogen fractions were reduced with 10 μL dithiothreitol (DTT) and gel electrophoresed under denaturing conditions with SDS Tris–glycine buffer, pH 8.5 at 150 V, 500 mA, for 90 min. Gel electrophoresis of BSA was performed using the native (nonreduced) protein at the same pH, current, and voltage conditions. Protein bands were then stained with Coomassie Brilliant Blue G-250 stain and washed with 10% acetic acid to reduce background staining.

Membranes showing differences in the ability to transfer proteins were further assessed using purified protein solutions, containing either BSA (2 mg mL^{-1}) or bovine thyroglobulin (669 kDa, 1 mg mL^{-1}), to examine the differences between the network membranes. To provide qualitative assessment of protein transfer, gel electrophoresis was performed as described above with fractions taken at 30-min intervals. Protein transfer was quantified using UV–vis.

Determination of protein transfer using UV–vis

For UV–visible spectrophotometry tests, a protein solution (15 mL) containing 40 m*M* Tris–glycine buffer and BSA (2 mg mL^{-1}), bovine fibrinogen (1 mg mL^{-1}), or bovine thyroglobulin (1 mg mL^{-1}) was placed in stream 1, and stream 2 was filled with 10 mL buffer. Samples were electrophoresed using the BF200 unit and 1.5-mL fractions were taken from the stream 1 and stream 2 reservoirs at 30-min intervals and simultaneously the volume in both the stream 1 and stream 2 reservoirs was recorded. Protein samples (1.0 mL) were diluted to 3 mL with buffer and the absorbance was measured at a fixed wavelength of 280 nm. A serial dilution of protein concentration in buffer was used to provide a standard curve of absorbance versus concentration percentage as a linear regression. To ensure the effects of electroendosmosis were accounted for, the amount of protein was determined using volume correction for both the stream 1 and stream 2 reservoirs. Beer's law was then used to quantify protein concentration based on this curve. These

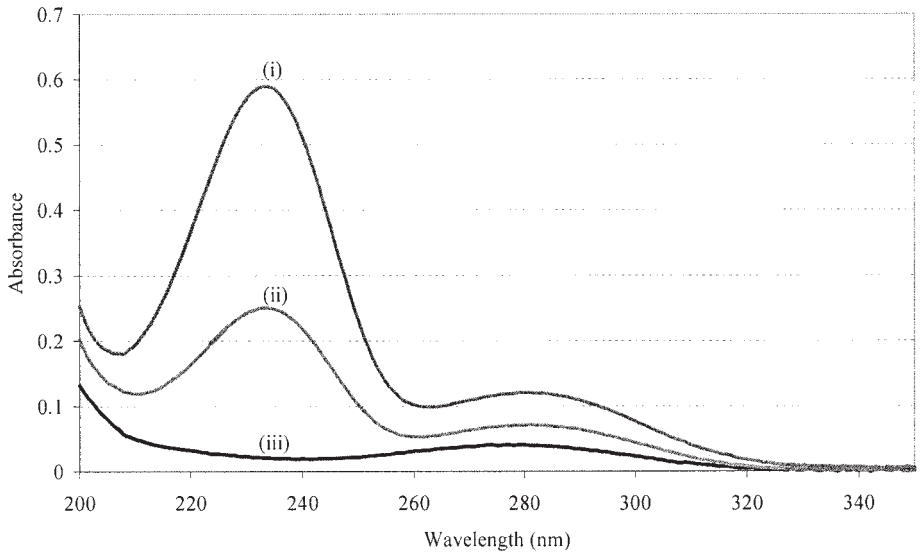

Figure 2 Ultraviolet absorption spectra of 0.1% (w/v) "glutaraldehyde" in distilled water as (i) commercial, (ii) treated with activated charcoal, and (iii) distilled solutions.

transfer experiments were performed in triplicate and the mean protein concentration was used to calculate average yield. Yield was determined by dividing the amount of protein in the stream 2 reservoir by the initial amount of protein in the stream 1 reservoir, and is shown as a percentage.

RESULTS AND DISCUSSION

Characterization of glutaraldehyde solutions

In attempts to describe "glutaraldehyde" solutions, authors have characterized "glutaraldehyde" in terms of purity.[1,2,8–11] Although this is correct to describe freshly distilled glutaraldehyde solutions, it has also been used to assess commercial-grade "glutaraldehyde" solutions.[8,9,16] The term "purity" implies the solutions contain aldehyde functional groups derived solely from the glutaraldehyde monomer and in our view should not be applied to commercial samples. The change in aldehyde content is a measure of oligomerization (see below).

Even though ^{1}H-NMR is convenient for most sample analyses, the spectra for glutaraldehyde in aqueous solutions were complex and, consequently, ultraviolet–visible (UV–vis) spectroscopy was chosen for further sample analysis. UV–vis is conveniently used to examine commercial-grade "glutaraldehyde" to assess the species present in the samples. Higher molecular weight aldol condensation oligomers of glutaraldehyde were observed using UV–vis (Fig. 2), where strong absorption at 235 nm for carbon–carbon double bond π–π^* transition of α,β-unsaturated aldehyde structures is seen, as reported previously.[16,26] This peak is particularly strong in commercial samples relative to the aldehyde peak for the n–π^* transition at approximately 280 nm. Treatment with activated charcoal decreases the oligomer content, observed as a decrease in intensity at 235 nm. Distillation leads to separation of glutaraldehyde from the residual oligomeric species.

Based on UV–vis assessment in this and other studies for simplicity, the major structures considered present in commercial-grade "glutaraldehyde" solution correspond to the glutaraldehyde monomer, its dimer, trimer, and tetramer aldol condensation products. The presence of both the dimer and trimer was confirmed in solution using mass spectrometry with $M^+ \cdot$ at *m/z* 183.16 (68%) and *m/z* 265.25 (10%), respectively.

The total aldehyde content determined for each solution used is given in Table I. This shows the aldehyde content of the commercial-grade glutaraldehyde solution was lower than that of the distilled glutaraldehyde solution. Freshly distilled product was calculated to be 99.9% glutaraldehyde according to Anderson's method.[8] The commercial-grade solution was determined to have significantly lower aldehyde content, showing that the commercial sample contains not only the monomer, but also a mixture of glutaraldehyde and other species.

TABLE I
Aldehyde Content as Determined by Acid Titration of Base Liberated from the Reaction of Known Weight of "Glutaraldehyde" Solutions with Bisulfite

"Glutaraldehyde"	Aldehyde (mmol g^{-1}) sample
Commercial	$15.67 \pm 7 \times 10^{-2}$
Distilled	$19.96 \pm 7 \times 10^{-2}$

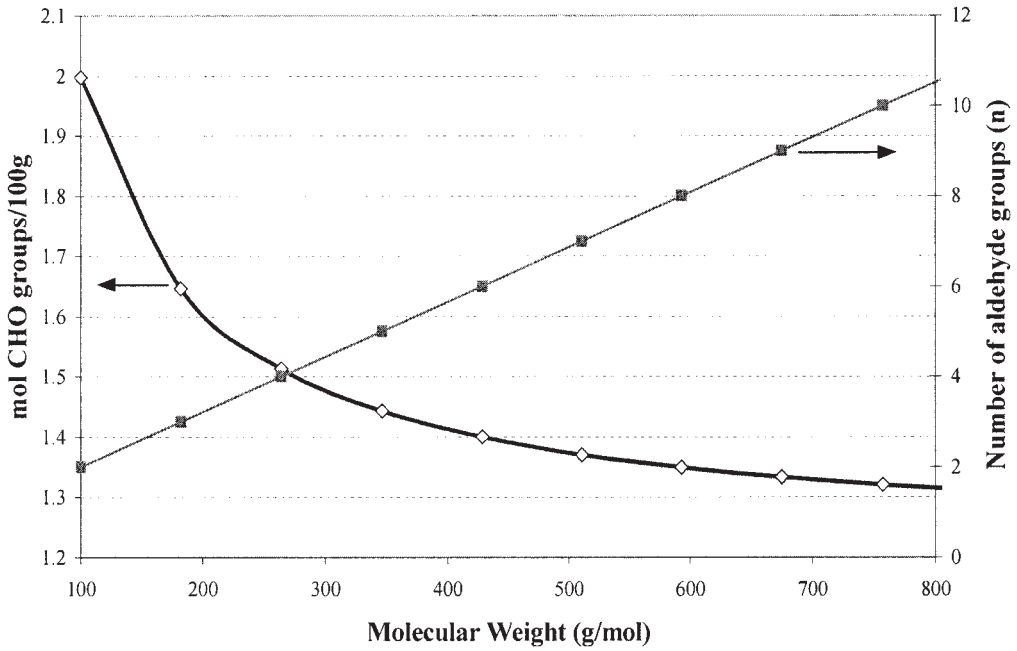

Figure 3 Relationship between number of aldehyde groups and molecular weight of glutaraldehyde and some of the possible aldol condensation oligomers.

To gain a better understanding for application in polymeric systems it is essential to be able to visualize the molecular structure of components and their reactive functional groups. Thus knowledge of functionality is necessary. The potential functionality of a molecule is defined to be the number of other molecules the compound has the potential to react with.[27] The actual functionality differs in that it refers to the actual number of sites that have reacted in the system being considered. The functionality of both molecules in a reaction is consequently controlled by the reaction stoichiometry.

By considering the number of aldehyde groups obtained by titration and molecular weight of the monomer glutaraldehyde and its respective oligomers, a linear relationship is obtained (Fig. 3). From this the equivalent number of aldehyde groups per 100 g of sample was reached by treating each molecular weight as a discrete entity (Fig. 3). Using this with the total aldehyde titration, the average potential functionality for each solution was determined. The freshly distilled solution was determined to have a potential functionality consistent with an equivalent of 2.00 aldehyde groups, with an equivalent average molecular weight of 100.02 g mol^{-1}.

Oligomerized "glutaraldehyde" has fewer aldehyde groups per gram because the number of functional groups in the solution decreases by one with each condensation. At the same time the average functionality of the molecule is increased. The commercial sample was determined to have a potential functionality consistent with an equivalent of 3.67 aldehyde groups, with an equivalent average molecular weight of 294.42 g mol^{-1}.

Network structure

Although PVA forms a physical hydrogel network through inter- and intramolecular interactions, the polymer can be crosslinked to establish a covalently linked network using crosslinkers reactive toward its functional groups. The chemical reactions involved in forming gels using glutaraldehyde were shown previously.[25] The current work investigated the effect of crosslinker and polymer concentration on the structure of PVA–glutaraldehyde hydrogel networks as well as the effect of the crosslinker source. Useful membranes were formed from glutaraldehyde : PVA1 at ratios that equate to dialdehyde : 1,3-diol moieties on the polymer from 1 : 25 to 1 : 6.25 between 5 and 20% PVA (w/v). The 1,3-diol is determined to be the total number of hydroxyl groups in the polymer chain divided by 2.

Effect of polymer concentration on network structure

The system chosen for study represents an extreme in terms of polymer formation and the effect that each of the components has on the overall system. Thus (in terms of functional groups) PVA–glutaraldehyde membranes use a vast excess of hydroxyl functional groups in the system from the PVA, which has high potential functionality. In other words the PVA is

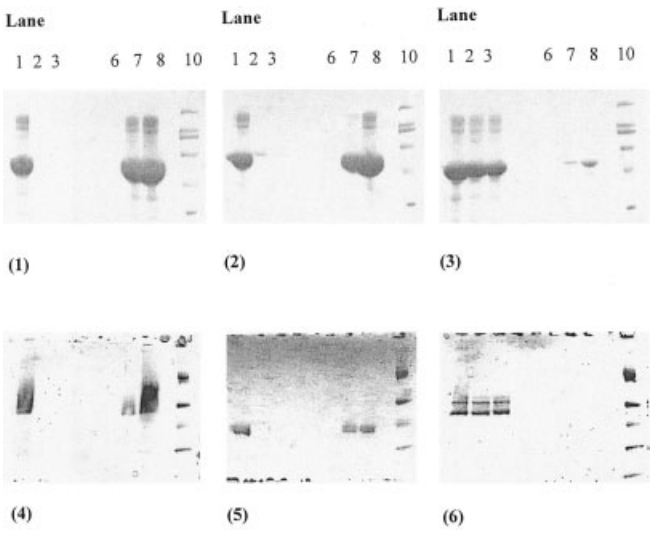

Figure 4 Protein transfer of BSA (1–3) through PVA membranes* crosslinked with glutaraldehyde at 1 : 18.75 dialdehyde to 1,3-diol ratio (1) 12.5%, (2) 15%, and (3) 17.5% (w/v) and fibrinogen (4–6) through PVA membranes crosslinked with glutaraldehyde at 1 : 25 dialdehyde to 1,3-diol (4) 5%, (5) 10%, and (6) 20% PVA (w/v). (*Electropherograms: lanes 1–3, stream 1; lanes 6–8, stream 2 fractions taken at 30-min intervals from start; lane 10, molecular weight marker.)

present in large excess even at higher levels of glutaraldehyde.

The effect of polymer concentration on the network structure was examined using preparative electrophoresis to assess protein transfer across the membranes at a fixed ratio of dialdehyde to 1,3-diol moieties with various polymer concentrations (Fig. 4). Electropherograms of BSA transferred across the membranes showed protein yield was decreased as the polymer concentration was increased.

Similar behavior was observed for a larger protein, fibrinogen (340 kDa). To facilitate visualization the actual protein has been reduced and denatured before gradient gel electrophoresis. Subsequently, the smaller fragments were observed in the electropherograms shown in Figure 4. It currently remains difficult to transfer fibrinogen through the available commercial poly(acrylamide) membranes for preparative electrophoresis applications.[28] In contrast, Figure 4 case (4) clearly shows the PVA–glutaraldehyde membranes to have successful transfer of bovine fibrinogen to stream 2. This indicates that the pore size is sufficiently large to allow the protein to pass through the network, which may be beneficial for the separation of such proteins from viruses in plasma solutions.

Quantitative analysis was carried out using the data in Table II of protein yield in stream 2, plotted against polymer concentration at a 1 : 25 ratio of crosslinker to 1,3-diol (Fig. 5). The axes show yield of protein as a percentage versus PVA concentration at a crosslink ratio of 1 : 25 dialdehyde to 1,3-diol. Curve (i) shows that as the concentration of polymer was increased at a constant crosslink ratio, the yield of BSA decreased.

Transfer of a larger globular protein, thyroglobulin (669 kDa) was also quantified and the decrease in yield was observed for this protein as the concentration of PVA was increased. Curve (ii) shows the yield change of thyroglobulin to be more significant than that found for BSA. This suggests that transfer through the network also relies on the size of the protein. For thyroglobulin the yield was found to fall to zero between 10 and 15% PVA (w/v), whereas BSA demonstrated successful transfer at both PVA concentrations. This indicates a molecular weight "cutoff" for the average pore size in the networks to be between

TABLE II
Electrophoresis Results for Transfer of BSA Across PVA–Glutaraldehyde Membranes Prepared Using Freshly Distilled Glutaraldehyde Solution After 30 min[a]

PVA % (w/v)	5%	5%	10%	10%	15%	15%	15%	20%	20%
Crosslink ratio	1 : 25	1 : 6.25	1 : 25	1 : 12.5	1 : 25	1 : 18.8	1 : 12.5	1 : 50	1 : 25
EEO (mL min^{-1})									
No BSA (20 min)	0.005	0.015	0.0075	0.005	0.005	0.01	0.01	0.005	0.01
+ BSA (1 mg mL^{-1})	0.016	0.016	0.029	0.016	0.025	0.016	0.05	0.033	0.033
% Transfer	99.6	99.4	97.9	98.1	98.8	97.7	98.6	98.7	33.0
	99.6	99.5	99.2	98.8	99.0	98.2	31.2	59.8	40.0
	99.6	99.7	98.6	97.7	98.6	98.7	33.0	58.5	35.6
Avg % Transfer	99.6	99.5	98.6	98.2	99.0	98.6	28.4	60.3	37.8
% Recovery	85.5	75.2	69.5	75.6	58.8	54.9	75.8	71.2	85.9
	83.7	75.1	76.7	73.2	64.1	55.5	73.7	79.6	82.2
	89.5	76.2	72.7	69.4	67.1	55.9	78.7	83.8	84.9
Avg % Recovery	86.2	75.5	73.0	72.7	63.3	55.5	76.0	78.2	84.3
% Yield	85.2	74.8	68.1	74.2	58.6	54.3	15.8	44.9	32.5
	83.3	74.7	76.1	72.3	63.5	54.5	23.0	47.6	32.9
	89.1	76.0	71.7	67.8	66.2	55.2	25.9	49.0	30.3
Avg % Yield	85.9	75.2	71.9	71.4	62.7	54.7	21.6	47.2	31.9

[a] As homogeneous solution in 40 m*M* Tris–glycine buffer, BSA (2 mg mL^{-1}).

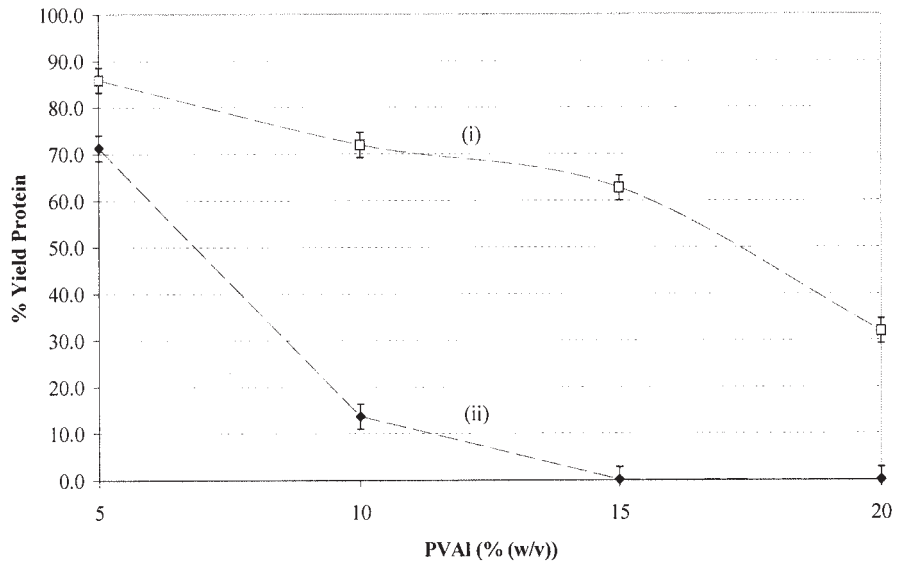

Figure 5 Average yield (i) BSA and (ii) thyroglobulin through PVA–glutaraldehyde membranes crosslinked at a ratio of 1 : 25 glutaraldehyde per 1,3-diol.

the size of BSA and thyroglobulin at this polymer concentration when crosslinked at this ratio (Fig. 5). Further evidence of molecular weight cutoff in networks was demonstrated for membranes prepared from PVA [10% (w/v)] crosslinked at a ratio of 1 : 12.5 glutaraldehyde per 1,3-diol; these also prevented transfer of thyroglobulin across the membranes.

Effect of crosslinker concentration on network structure

Gel structure morphology was examined using cryogenic SEM to prevent collapse of the gel network on drying. Figure 6 shows SEM microphotographs of PVA gels crosslinked at the same polymer concentration and different crosslink densities. Gel (1) is made from 5% PVA (w/v) crosslinked with glutaraldehyde at a ratio of 1 : 25 dialdehyde to 1,3-diol moiety on the polymer. Gel (4) was prepared at the same PVA concentration at a higher ratio of 1 : 6.25 dialdehyde to 1,3-diol moieties. Both gel networks were observed to have uniform pores and are clearly different from one another. A clear mesh is observed for (1); in comparison (4) shows a more highly crosslinked network where the mesh is less distinguishable and the network appears to have smaller pore size at the higher crosslinker concentration.

The amount of water absorbed by the dried PVA gel networks was measured. Lower swelling ratios typify tighter networks that can be interpreted as having an overall lower porosity. The water-swelling behavior observed shows a trend that, as the level of crosslinker is increased, the water absorbed by the network is decreased, as shown in Figure 7. After 120 min swelling in water a gel prepared at a ratio of 1:25 dialdehyde to 1,3-diol was found to swell 136% more (1.5/1.1) than the corresponding gel made at the higher crosslinker ratio of 1 : 6.25.

Preparative membrane electrophoresis was used to further compare the network properties.[25] Gradient gel electrophoresis was used to aid visualization of protein transfer from stream 1 to stream 2. The observed successful transfer of BSA for all networks assessed suggests that the effective pore size of each membrane network exceeds the 67-kDa size of BSA for a constant polymer concentration of PVA [5% (w/v)] crosslinked with distilled glutaraldehyde at various dialdehyde to 1,3-diol ratios. It was also found that as the number of crosslinks per polymer chain increase at a constant polymer concentration, qualitatively the amount of protein transferred to stream 2 decreased, as shown in Figure 8.

UV–vis was used to quantify the protein transfer across membranes, taking into account volume change arising from electroendosmosis. Table II shows the yield, recovery, and transfer of BSA across PVA–glutaraldehyde membranes prepared at various concentrations of polymer and crosslinker. The yield of protein in stream 2 was determined as a percentage of the initial total protein in the system. From these data the protein yield in stream 2 was plotted against the ratio of dialdehyde to 1,3-diol moieties.

Quantitative protein yield trends were found to be similar to those observed by gel electrophoresis (Fig. 9). As the number of crosslinks increased at constant PVA concentration, the protein yield decreased, indicating an overall tighter network with smaller pore size. It was also noted that at low PVA concentration the difference in protein yield is low, whereas at high

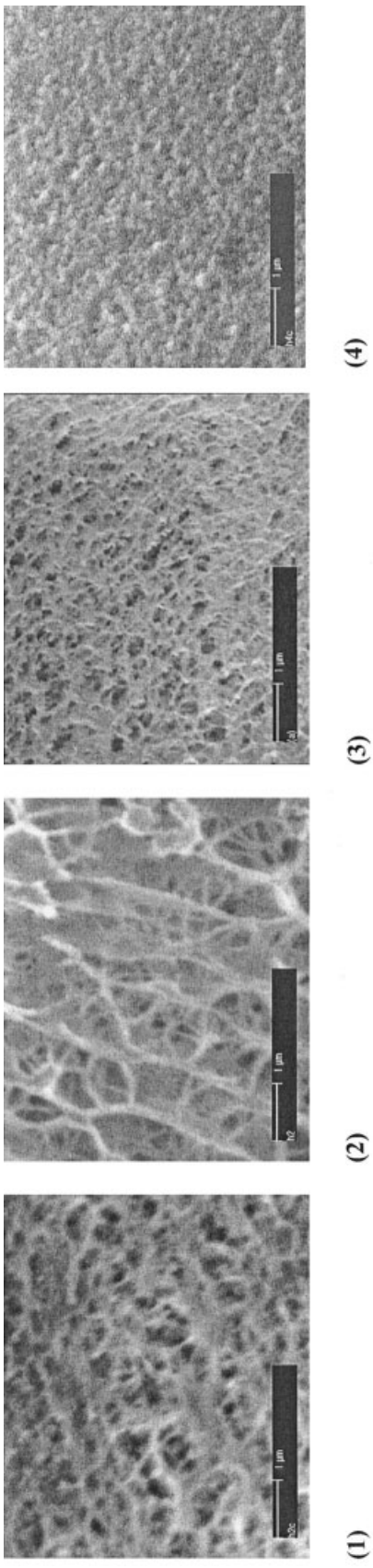

Figure 6 SEM images (magnification ×15,000) obtained for (1) 5% (w/v) PVA crosslinked gel with fresh glutaraldehyde at 1 : 25, (2) using the same weight commercial glutaraldehyde and (3) using the equivalent of commercial glutaraldehyde based on aldehyde concentration, and (4) 5% (w/v) PVA crosslinked gel with distilled glutaraldehyde at 1 : 6.25.

PVA concentrations the difference in protein yield becomes significantly larger. This suggests that the effect of high crosslinker concentration on the network is further compounded when combined with higher polymer concentration.

Effect of "glutaraldehyde" source on network structure

For simplicity this discussion will focus further on a comparison of two species, glutaraldehyde (the monomer) and the dimer. The latter has three aldehyde groups. However, in the experimental section, results for other oligomers of glutaraldehyde have been included to establish the general nature of this work.

Two scenarios for glutaraldehyde use are considered: (1) use of a given weight of the reagent "out of the bottle" (i.e., the weight of "glutaraldehyde" is assumed to be pure glutaraldehyde, the most common approach) and (2) use of aldehyde titration to adjust the amount of oligomer required based on aldehyde concentration. The control in all the above is glutaraldehyde [Fig. 1(i)]. The term glutaraldehyde will be used to describe the monomer, and dimer refers to the compound shown in Figure 1(ii).

Substitution of glutaraldehyde on a weight basis out of the bottle

A comparison of networks using SEM, swelling, and electrophoresis data when glutaraldehyde is replaced by the same weight of commercial glutaraldehyde solution (out of the bottle) reveals an interesting difference. The latter have larger pore sizes, as established using SEM [Fig. 6, (1) and (2)]. The swelling behavior for 5% (w/v) PVA gels crosslinked with the same amount of commercial-grade glutaraldehyde solution was also consistently higher than that for those prepared using the distilled crosslinker, indicating the latter to have a smaller network pore size (Fig. 7). This is not the expected result if only the potential functionality of the aldehyde entity is considered. In the specific case of the trifunctional dimer, the outcome is counterintuitive.

The structures that result from (i) the reaction of the 2 moles of glutaraldehyde, on the one hand, and (ii) 1 mole of dimer, on the other, are shown schematically in Figure 10. The crosslinkage by the 2 moles of glutaraldehyde will use up 8 moles of hydroxyl groups from the polymer, whereas in the second scenario the dimer reaction will consume 6 moles of hydroxyl groups. Despite the higher functionality of the dimer crosslinking entity, it can be seen that in terms of functionality the systems are equivalent, given that both incorporate 3 moles of PVA. Thus on the same weight basis as the functionality of the "glutaraldehyde" mer is increased, the number of PVA chains

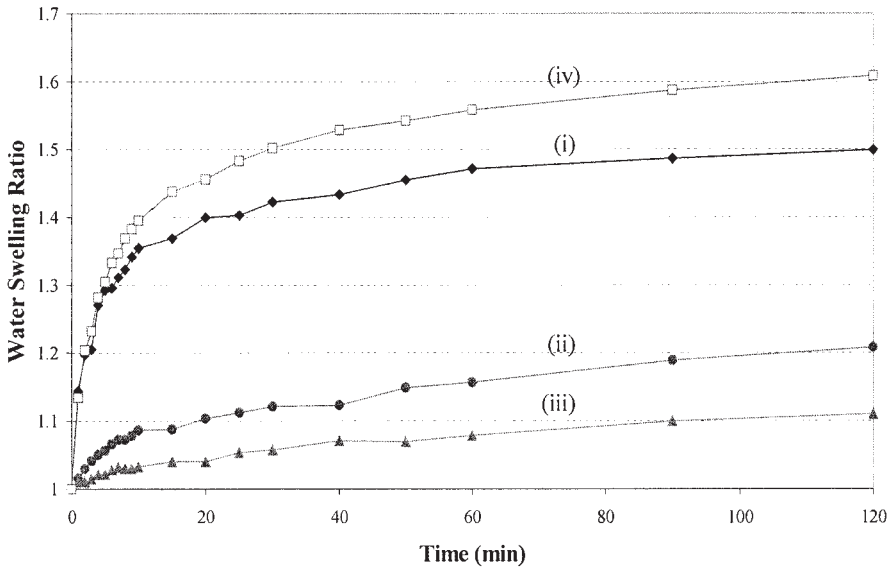

Figure 7 Water swelling ratio of 5% (w/v) PVA crosslinked gel with glutaraldehyde at (i) 1 : 25, (ii) 1 : 12.5, (iii) 1 : 6.25, and (iv) 1 : 25, same weight of commercial solution.

incorporated into the network remains the same. The dimer does not increase the amount of crosslinking. On the contrary, it leads to a more branched, starlike network, as shown in Figure 10(ii). This is characterized to be a looser network, as evidenced in this study. Similarly, the use of higher oligomers increases the starlike structure. Thus under the conditions of a large excess of PVA and the use of the same weight of aldehyde solution the oligomers give a more starlike network, not a more crosslinked structure. The functionality of the overall system is not altered, irrespective of the degree of oligomerization of the glutaraldehyde source. This conclusion is supported by the experimental results where the starlike structure corresponds to the observation of larger pore size.

Substitution of glutaraldehyde on an equivalent aldehyde basis

The titration for aldehyde in our view is a measure of the degree of oligomerization and reflects the average molecular weight of oligomers in solution. Although previously interpreted to represent a measure of purity of glutaraldehyde, UV absorbance and network results suggest the structures to be the outcome of a mixture of aldehydic species in the reagent with higher average functionality than that of glutaraldehyde.

When the titrated aldehyde concentration is used to keep the reaction stoichiometry the same (i.e., equivalent number of aldehydes used), the result is the functionality of the crosslinking entity and the system as a whole is increased. In the case of the dimer 1 1/3 as much dimer would be required to replace 2 moles of glutaraldehyde. On this equivalent aldehyde basis, the dimer can crosslink more PVA chains than on the same weight basis.

The difference in the networks is consistent with the SEM results from this (Fig. 6, (1) and (3)]. This is also evidenced electrophoretically where a tighter overall network pore structure was observed qualitatively using protein transfer of BSA (Fig. 11). The reduced transfer of BSA from stream 1 to stream 2 also evidences this for the membranes prepared using the equivalent aldehyde concentration of commercial-grade glutaraldehyde solution. For example (1) partial protein transfer was observed for a PVA (15% w/v) membrane prepared using the distilled glutaraldehyde crosslinked at a 1 : 18.75 dialdehyde to 1,3-diol ratio. In comparison, no protein was observed to have been transferred to stream 2 for the membrane prepared using the equivalent of commercial glutaraldehyde, (2). A similar trend was also found using fibrinogen, whereby partial transfer of the protein was observed for a PVA (10% w/v) membrane crosslinked at a ratio of 1 : 12.5 using the distilled glutaraldehyde

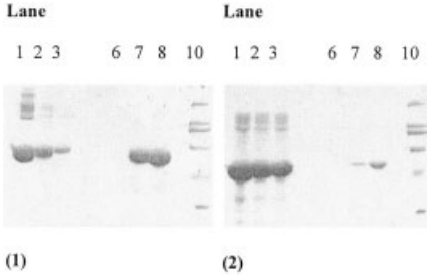

Figure 8 Protein transfer of BSA through PVA (17.5% w/v) membranes* crosslinked with glutaraldehyde at (1) 1 : 25 and (2) 1 : 18.75 dialdehyde to 1,3-diol ratio. (*Electropherograms: lanes 1–3, stream 1; lanes 6–8, stream 2 fractions taken at 30-min intervals from start; lane 10, molecular weight marker.)

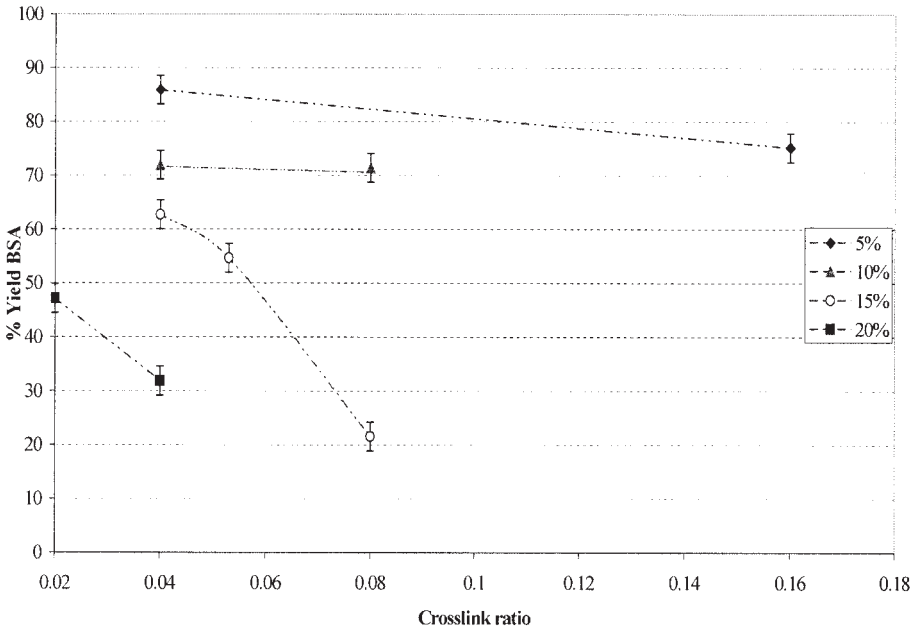

Figure 9 Average-yield BSA at various crosslink ratios for PVA membranes at different concentrations [% (w/v)].

solution. In comparison, no protein was found to have been transferred to stream 2 for the membrane prepared using the equivalent amount of commercial glutaraldehyde based on aldehyde concentration.

Comparison of qualitative protein transfer across selected membranes at critical crosslinkage provided further evidence of tighter pore sizes for various networks at different PVA and glutaraldehyde concentrations, prepared using the equivalent amount of commercial glutaraldehyde based on aldehyde concentration than the corresponding membranes prepared using distilled glutaraldehyde.

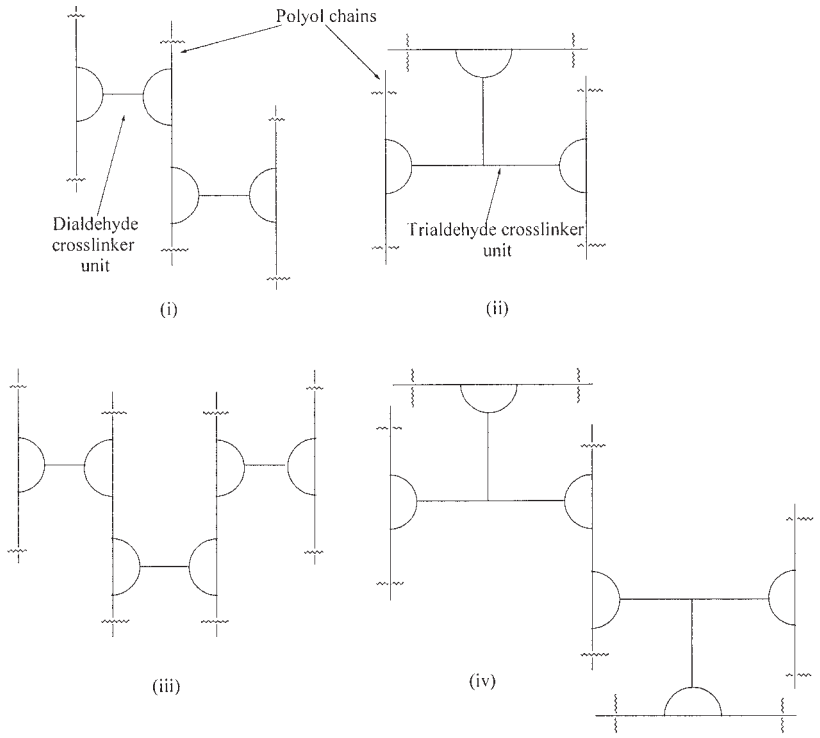

Figure 10 Schematic representation of crosslinkage of (i) three polyol chains with two dialdehyde molecules, (ii) three polyol chains with the same weight of dimeric trialdehyde (one molecule), (iii) four polyol chains with three dialdehyde molecules, and (iv) five polyol chains with the equivalent amount of dimeric trialdehyde (two molecules).

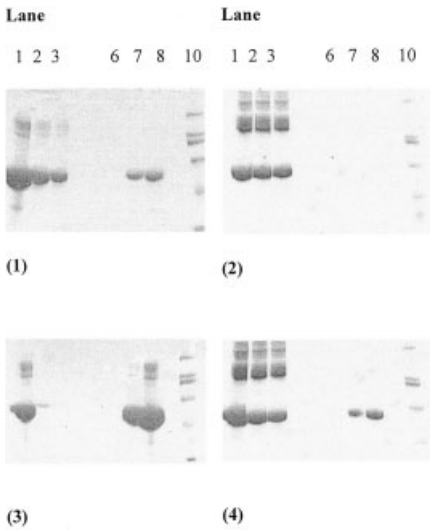

Figure 11 Protein transfer of BSA across PVA (15% w/v) membranes* crosslinked at 1 : 12.5 (1 and 2) and 1 : 18.75 (3 and 4) dialdehyde to 1,3-diol ratio with (1 and 3) distilled glutaraldehyde and (2 and 4) the equivalent of commercial glutaraldehyde based on aldehyde content. (*Electropherograms: lanes 1–3, stream 1; lanes 6–8, stream 2 fractions taken at 30-min intervals from start; lane 10, molecular weight marker.)

Figure 10(iii) illustrates the structure obtained using three pure glutaraldehyde units compared with the equivalent network formed using the aldehyde equivalent of dimer (iv). Here, the glutaraldehyde units (six aldehydes) can link four PVA chains. In comparison, two dimer units (six aldehydes) can increase the incorporation of PVA by linking five chains, a point not fully recognized. When substituted on an equivalent aldehyde basis the higher oligomers of glutaraldehyde increase the complexity of the network structure and give more crosslinked and more highly branched structures. These oligomers have the potential to increase the crosslink density in the network as the number of branch points in the oligomers increases with each condensation to higher oligomer, thus generating a tighter network structure as observed in this study (Fig. 6).

Theoretical calculation of gel point in other glutaraldehyde systems

The same reasoning applies to other uses of glutaraldehyde, such as in protein fixation where in the reaction essentially amine groups of proteins are reacted with aldehyde groups of the crosslinker.

The average functionality of a system is defined using classical gelation theory to be the equivalents that can react divided by the total moles present.[29–34] In step-growth reactions actual functionality is controlled by stoichiometry, a point not fully recognized by some of the previous workers. This allows the growth of the polymer to be controlled.

Initially developed by Carothers, using balanced stoichiometric amounts of reactants, gel point calculations are used to approximate the extent of reaction at which an infinite network is formed.[33,34] This method for approximation of the gel point (p_{gel}), based on number-average molecular weight, is simple for determining the number average for functionality (F_{avg}) and is used to give comparative data. For simplicity it is convenient to illustrate F_{avg} and p_{gel} with modification of the Carothers method as reported by Pinner and Solomon.[29,30] This takes into account that the extent of oligomerization depends on the limiting reactant because the reactant in excess actually reduces the system functionality. This method has been applied in the present work to examine gelation where the glutaraldehyde or its dimer is used with a close to balanced stoichiometry in reactions with a potentially bifunctional reagent.

Table III shows the theoretical gel point calculations for systems where glutaraldehyde, its dimer, trimer, or a mixture of dimer and trimer are reacted with a simple diamine. Where $F_{avg} \leq 2$, gelation does not occur ($p_{gel} \geq 1$), as demonstrated for reaction between 2 mol of the dialdehyde with 2 mol of the diamine. Gel point calculations for a simple diamine system, such as ethylenediamine at balanced stoichiometry, behave as classical 2 : 2 functional systems in the case of glutaraldehyde, as expected, giving rise to a linear structure (Table III).[33,34] Excess of either component restricts the molecular weight of the resultant polymer and reduced the actual functionality of the other component.[31,35]

Similar results are observed for systems where the same aliquot weights of its higher oligomers are reacted. When the dimer replaces glutaraldehyde on a weight basis, intuitively given that the dimer is potentially trifunctional, a network would be expected to form. However, there is excess amine (Table III) and taking into account the stoichiometry of the system the resultant structure is restricted in size and does not lead to gelation because the value of F_{avg} is 2. Further branching is introduced when trimer (potential functionality of 4) replaces glutaraldehyde, although F_{avg} is still 2. The degree of oligomerization in a balanced formula does not alter the functionality of the system, it alters branching. Use of higher-functionality crosslinkers would be expected to lead to more star-shaped structures, which are also found in networks prepared by chain-growth methods.[36]

In comparison, when the stoichiometry of groups able to react in the system is balanced, higher oligomers show that gelation of the network will result ($F_{avg} > 2$) and, as expected, when the aldehyde number (functionality) of the oligomer is increased, gelation will occur at an earlier extent of reaction. On an

TABLE III
Comparison of Same Aliquot Weight with Balanced Stoichiometry on Oligomerization Versus Gelation

Monomer (mer)	Mol mer	Mol CHO	Mol diamine	Mol NH_2	Mol lim. funct. grp.[a]	F_{avg}	p_{gel}
Dialdehyde (glutaraldehyde)	2	4	2	4	4	2	1
Trialdehyde (dimer)	1	3	2	4	3	2	1
Tetraldehyde (trimer)	2/3	2 2/3	2	4	2 2/3	2	1
Mixture of trialdehyde and tetraldehyde							
50 : 50	5/6	2 5/6	2	4	2 5/6	2	1
Dialdehyde	2	4	2	4	4	2	1
Trialdehyde	1 1/3	4	2	4	4	2 2/5	0.83
Tetraldehyde	1	4	2	4	4	2 2/3	0.75
Mixture of trialdehyde and tetraldehyde							
50 : 50	1 1/6	4	2	4	4	2 10/19	0.79

[a] Mol. lim. funct. grp. is defined to be the moles of the limiting reagent functional groups (either NH_2 or CHO) in the system.

equivalent aldehyde basis (i.e., the number of aldehyde groups in the system is kept constant) a classical 2:3 functionality system exists using the diamine and the dimer, so gelation is expected at 83% reaction (Table III). Higher oligomers will gel at even lower extent of reaction arising from the higher number of branch points in the crosslinking molecule as indicated.

CONCLUSIONS

Variation to the formulation of PVA–glutaraldehyde gel networks showed that the effect of increasing either the polymer concentration or the crosslinker concentration caused a decrease in the pore size of the network.

The use of different crosslinker sources enabled different networks to be made from PVA–glutaraldehyde. Those prepared using the same weight of commercial technical grade glutaraldehyde solution were found to have larger pores, whereas the equivalent amount on an aldehyde concentration basis had smaller pores than those of the corresponding network made with freshly distilled glutaraldehyde solution. This is attributable to the inclusion of oligomers of glutaraldehyde. It was shown that as the degree of oligomerization of the aldehyde increased, the network formed became more star-shaped (i.e., the dimer leads to a more branched network). Importantly, different degrees of incorporation of PVA are observed when the equivalent amount of dimer is used, based on aldehyde titration, to form a network.

The use of oligomers with higher potential functionality leads to branching, whereas the stoichiometry of the system (as with all step-growth reactions) defines whether simple branching with a restricted resultant structure or branching with network formation will result. The way to influence crosslinking in step-growth–prepared polymers is to take stoichiometry into account. Increasing functionality does not necessarily lead to the formation of tighter network structures; depending on stoichiometry, the result may be to reduce crosslinking. This was demonstrated for diamine systems where glutaraldehyde was replaced with higher-functional oligomers. In these systems the higher functionality is compensated by nonstoichiometric formulation. When the oligomeric aldehydes are used and system stoichiometry is maintained, actual functionality is increased.

Careful selection and characterization of crosslinkers used for network preparation are necessary. Using commercial "glutaraldehyde" solutions "out of the bottle" leads to variable products. These solutions contain a mixture of glutaraldehyde and oligomers where an average structure in solution is obtained. The use of commercial-grade glutaraldehyde to prepare membrane and fixation networks that require characterization can lead to problems with structure. It is recommended for reproducibility that glutaraldehyde must be isolated, purified, characterized, and used as quickly as possible. Failure to do so leads to variation in molecular structure. In these systems it is imperative that the correct stoichiometric amounts of reagent are added. Unlike chain-growth systems, adding more or less reagent in a step-growth reaction can lower the system's actual functionality and thus reduce the chances of gelation.

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