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Role of the structural parameters of the macromolecular matrix in polymer-supported peptide synthesis: attempts at optimization

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Investigations into the optimization of the structural parameters of the macromolecular matrix with a view to designing efficient polymer supports for peptide synthesis are reported. The dependence on the chemical nature of the monomers, nature and extent of crosslinking, the method of polymerization, hydrophobic–hydrophilic balance of the matrix, its chemical and mechanical stability, ease of functionalization, swelling/solvation behaviour, porosity and pore dimensions of the resin particles, and the chemical reactivity of the attached functional moiety on the variables of polymer synthesis are delineated. Acrylamide was crosslinked with highly hydrophilic tetraethyleneglycol diacrylate and triethyleneglycol dimethacrylate through medium polar *N,N'*-methylene-bis-acrylamide to highly hydrophobic and rigid divinylbenzene. These copoly(acrylamide)s were amino-functionalized by polymer-analogous reactions. The extent of functionalization, reactivity, porous nature, solvent compatibility and thermal behaviour of these resins was found to be dependent on the fine molecular character as well as on the nature and extent of crosslinking.

(Keywords: poly(acrylamide)s: functionalization; crosslinking)

INTRODUCTION

The advances achieved in solid phase peptide synthesis, originally introduced by Merifield in 1963[1], have opened up new vistas in the multi-step synthesis of chain molecules with predetermined amino acid sequences. The solid phase synthesis has been proved to be exceptionally useful in the synthesis of polypeptides with marked biological activity[2]. The solid support is a synthetic crosslinked polymer possessing reactive functional sites which covalently attach the amino acids to them. The physicochemical properties of the polymer support dictate its suitability for efficient gel-phase reactions[3]. It must permit rapid, unhindered contact between the growing peptide chain and the low molecular weight reagents. It must be readily separable from the solution phase at every stage of the synthesis and must be physically stable under these changing conditions of operation[4].

Although crosslinked polystyrene-based resins are commonly used as supports[5–7], their relative hydrophobic character, in comparison with the more polar organic media required to solubilize reactions, becomes problematic in the early stages of chain assembly, where the resin-to-peptide mass ratio is high and the physical properties of the support dominate[8]. Systematic investigations by various groups have shown that the synthetic reactions are facilitated to the maximum when the polymer support and the peptide chains have comparable polarities[9–15]. More recently, peptide–resin conjugates have been synthesized on polar hydrophilic crosslinked aminoalkyl poly(dimethylacrylamide) supports for solid phase immunoassays[16]. In these polyamide-based supports, free and fair permeation of compatible solvents is possible due to the highly polar and porous nature. The parameter of polarity, in turn, is dependent on the polar nature of the monomers, the chemical nature and extent of crosslinking agents, changing experimental conditions, method of polymerization, hydrophilic–hydrophobic balance of the support, reactivity of functional groups and distance of separation of the functional groups from the polymer backbone, and porosity and surface morphology of the polymer. These factors were taken into consideration in the design of new polymer supports intended for peptide synthesis, which are reported in this paper. The thermal behaviour of the resins was also examined.

A proper understanding of the interdependence of functional group reactivity and molecular architecture of the polymer support is of contemporary interest in designing tailor-made polymers for specific applications[17–21].

EXPERIMENTAL

Materials and equipment

All reagents were of certified ACS grade. I.r. spectra were recorded on a Shimadzu IR 470 spectrophotometer. Scanning electron micrographs were recorded on a JSM

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Jeol-35 scanning electron microscope. Differential thermal analysis (d.t.a.) of the samples was carried out in a Leeds Northup DTA unit.

General procedures

Synthesis of crosslinked poly(acrylamide)s ***1(a–d)***. Acrylamide (6.74 g) and *N,N'*-methylene-bis-acrylamide (NNMBA) (0.78 g) were dissolved in water (75 ml) at 70°C. The solution of the monomer mixture was purged with nitrogen gas. Potassium peroxodisulfate (100 mg) was added to the monomer mixture and stirred at 70°C until the polymer was precipitated. Water (50 ml) was added to the polymer and heated at 80°C for 20 min. The lumps were powdered, filtered and the polymer was washed successively with water (20 ml, three times), ethanol (20 ml, three times) and finally with methanol (20 ml, three times for 3 min). The product resin was dried to constant weight in an air oven at 80°C. Crosslink densities were varied to 10, 15 and 20 mol% by varying the relative amounts of the monomers. Tetraethyleneglycol diacrylate (TEGDA) and triethyleneglycol dimethacrylate (TEGDMA) crosslinked (5–20%) poly(acrylamide)s **2(a–d)** and **3(a–d)** were prepared by solution polymerization of the monomers in ethanol at 70°C. Acrylamide–divinylbenzene (DVB) copolymers (5–20%), **4(a–d)** were prepared by benzoyl peroxide-initiated polymerization in ethanol. *Table 1* gives the details of the preparation of various crosslinked poly(acrylamide)s.

Amino functionalization of poly(acrylamide)s: preparation of poly(2N aminoethyl acrylamide)s and poly(2N aminohexyl acrylamide)s. Ethylenediamine (50 ml; 98%) was preheated in a glass vessel in an oil bath at 90–110°C. Powdered polymer **1a** (5 g) was added gradually with stirring to avoid formation of lumps. The mixture was heated under reflux for 9 h at 90–110°C. The reaction mixture was poured into water (500 ml) containing crushed ice. The resin was filtered and washed with sodium chloride solution (0.1 M) until the washings were amine-free, as indicated by a negative ninhydrin test. The gel was again washed with distilled water until free from chloride ions and finally with methanol for deswelling. The resin was dried under vacuum at 50°C to give 4.45 g of dry amino resin.

I.r. (KBr): 1650 cm^{-1} (amide), 3400 cm^{-1} (amine). Resins **1(a–d)**, **2(a–d)**, **3(a–d)** and **4(a–d)** were functionalized in a similar way. In the preparation of poly(2N-aminohexyl acrylamide)s, a solution of hexamethylene diamine in tetrahydrofuran (THF) was used for the transamidation reaction.

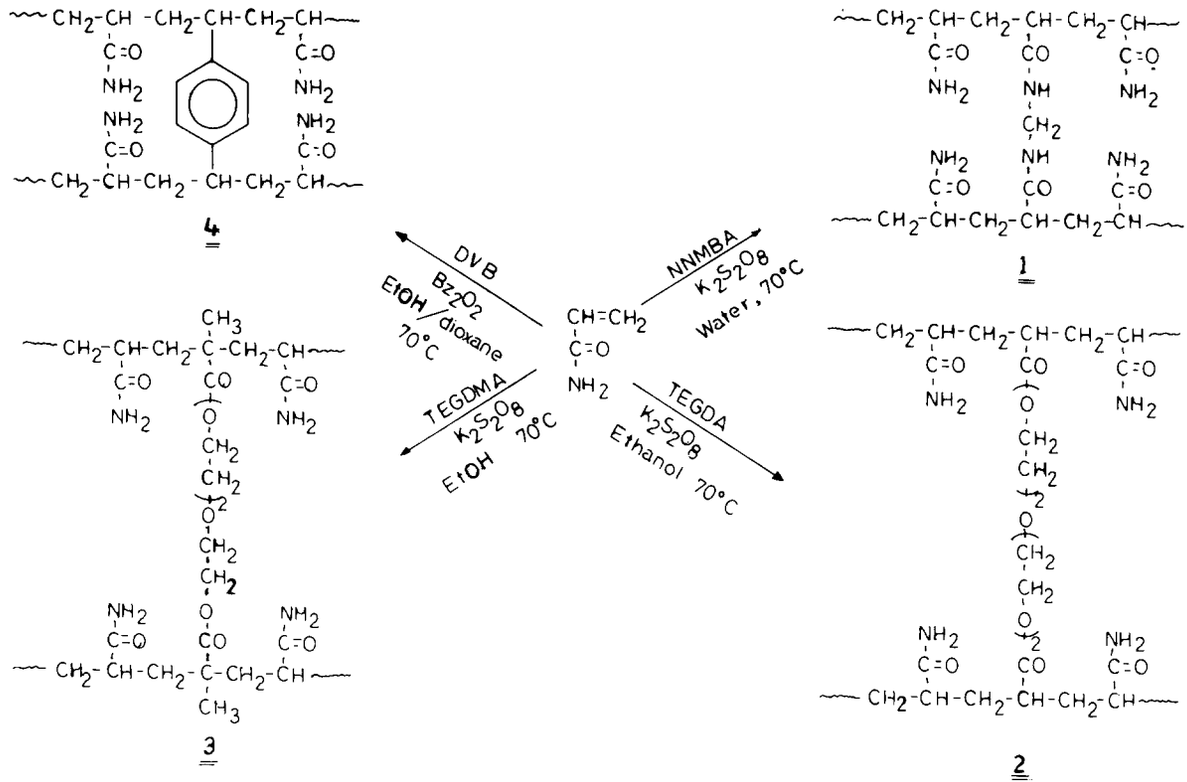
Estimation of amine content of the resin. The amino resin (200 mg) was stirred with hydrochloric acid (0.2 M, 10 ml) for 24 h, filtered, and washed with distilled water. The combined filtrate was titrated against NaOH (0.2 M) to a phenolphthalein end point. A blank was also performed. The amino capacity of all the resins derived from **1(a–d)**, **2(a–d)**, **3(a–d)** and **4(a–d)** was similarly determined.

Aminolysis of active esters by polymeric amines—assessment of efficiency in peptide bond formation. An accurately weighed sample of the amino resin (75 mmol) was stirred with an equimolar solution of *N*-benzoyl glycine 4-nitrophenyl ester in dioxane/water (1:1, 25 ml) for 5 h. The resin was filtered through a quantitative filter paper, washed repeatedly with distilled water until free from 4-nitrophenol, and dried. A known weight (100 mg) of the dry resin was stirred with HCl (0.2 M, 10 ml) for 24 h. The resin was filtered, washed free of HCl, and the combined washings were titrated against NaOH (0.2 M). The residual amino capacity was estimated, from which the extent of peptide coupling was obtained. Aminolysis reaction was carried out using all the poly(2N aminoethyl) and poly(2N aminohexyl) acrylamides.

Table 1 Preparation of various crosslinked poly(acrylamide)s

Sample code	Crosslinker	Extent of crosslinking (mol%)	Monomers used (g)		Yield (g)	Conversion[a] (%)
			Acrylamide	Crosslinker		
1a	NNMBA	5	6.74	0.78	5.85	77.9
1b	NNMBA	10	6.39	1.56	7.81	98.2
1c	NNMBA	15	6.03	2.34	7.86	93.9
1d	NNMBA	20	5.68	3.12	8.35	94.8
2a	TEGDA	5	6.74	1.51	7.40	89.6
2b	TEGDA	10	6.39	3.02	7.05	74.9
2c	TEGDA	15	6.03	4.53	8.07	76.4
2d	TEGDA	20	5.68	6.04	9.95	84.9
3a	TEGDMA	5	6.74	1.43	5.41	65.0
3b	TEGDMA	10	6.39	2.86	7.90	84.0
3c	TEGDMA	15	6.03	4.29	7.00	66.5
3d	TEGDMA	20	5.68	5.72	11.10	94.8
4a	DVB	5	6.74	0.65	6.57	88.9
4b	DVB	10	6.39	1.30	6.60	85.8
4c	DVB	15	6.03	1.95	6.72	84.2
4d	DVB	20	5.68	2.60	5.62	67.8

[a] Calculated on the basis of weights of monomers used



Scheme 1 Synthesis of copolymers of acrylamide

Polymer morphology

Polymer samples were sputtered with gold and scanned at an accelerated voltage of 15 kV in an electron microscope. They were micrographed at different magnifications which revealed the best surface details.

Thermal behaviour

Differential thermal analyses of the samples were carried out in a thermal analyser unit under nitrogen atmosphere. The reference used was alpha alumina. Samples were heated at the rate of 10°C min^{-1}.

Swelling characteristics

Dry polymer samples (initial weight W_i, 1 g) were allowed to swell to equilibrium point in various solvents employed in peptide synthesis. After 24 h, the resins were removed, surface water was carefully wiped off and the final swollen weight (W_f) determined. Percentage swelling of the resins in various solvents was calculated using the relation:

$$\text{Percentage swelling} = \frac{W_f - W_i}{W_i}$$

Crosslinked copolymers of acrylamide containing varying mole proportions of hydrophilic and hydrophobic crosslinking agents provide a polar support medium with optimum hydrophilic–hydrophobic balance for peptide synthesis. By varying the relative amounts of the monomers and the crosslinking agents and their nature, efficient polymeric supports can be designed. The support characteristics and the physicochemical properties of the polymer are dependent on a number of variables of polymer synthesis, such as the nature and extent of crosslinking, method of polymerization, degree of functionalization, hydrophilic–hydrophobic balance, porosity, swelling and solvation.

RESULTS AND DISCUSSION

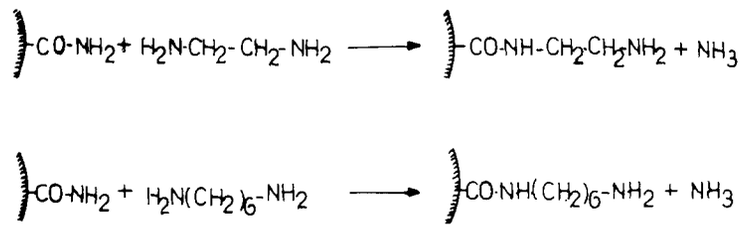
Synthesis of copolymers

Poly(acrylamide)s with varying mole proportions (5, 10, 15 and 20 mol%) of the crosslinking agents NNMBA, TEGDA, TEGDMA and DVB were prepared by solution polymerization of the monomers in the presence of free radical initiators (*Scheme 1*). These copolymeric resins differ widely in their hydrophilic–hydrophobic balance due to the difference in the nature and extent of crosslinking agents present in them. TEGDA and TEGDMA induce flexibility and hydrophilicity in the polymer matrix. DVB, on the other hand, is rigid and hydrophobic. NNMBA is intermediate in this range in inducing these properties.

Functionalization to amino resins

These copolymers were functionalized using a five-fold molar excess of ethylene diamine[22] or a THF solution of hexamethylene diamine (*Scheme 2*). These amino resins were characterized by i.r. spectroscopy and ninhydrin test[23]. The amino capacities of these resins were estimated by equilibrating a known weight of the amino resins with 0.2 M HCl and estimating the unreacted acid by alkali titration[24]. The variation in amino capacities of the different resins with the extent of crosslinking is depicted in *Figure 1*.

A definite dependence of amino capacities on the crosslink density and the hydrophilic nature of the crosslinking agent was observed. In poly(2N aminoethyl acrylamide)s crosslinked with NNMBA and DVB, amino



Scheme 2 Functionalization of crosslinked poly(acrylamide)s

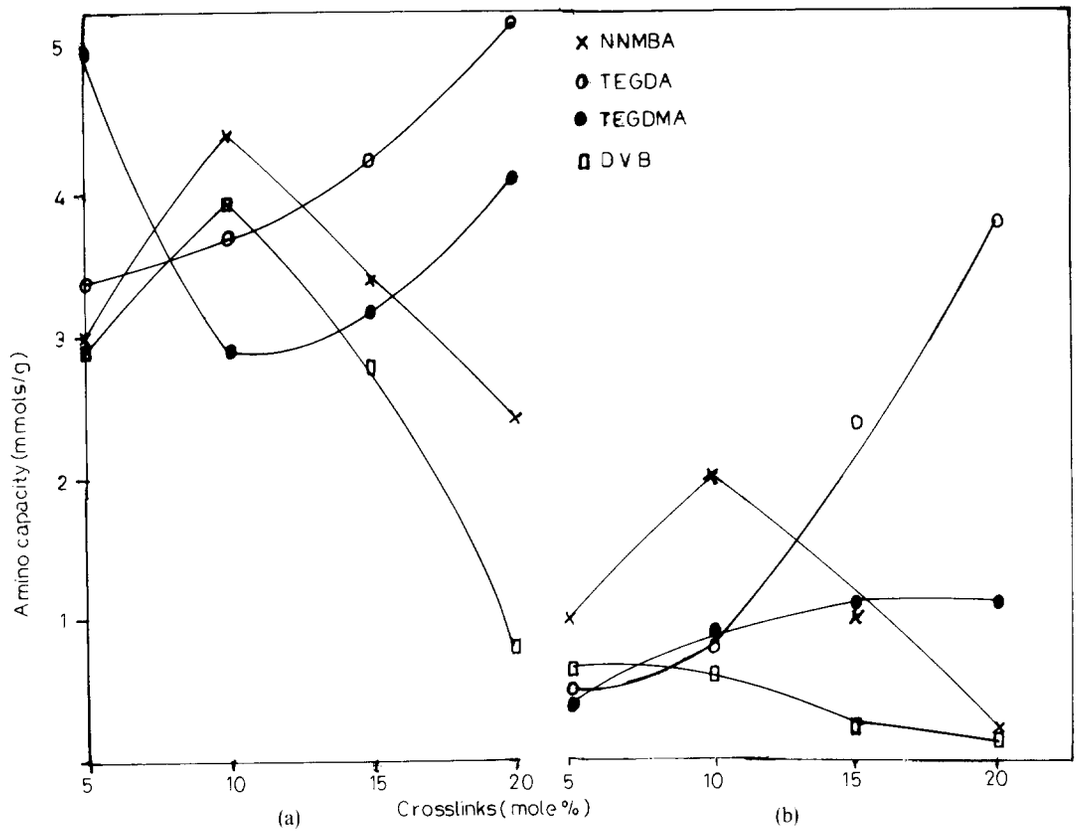
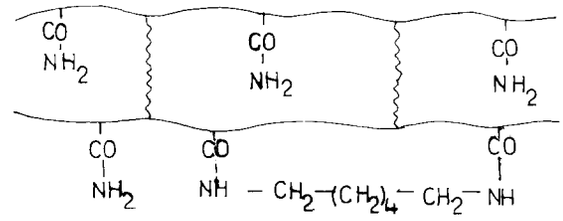


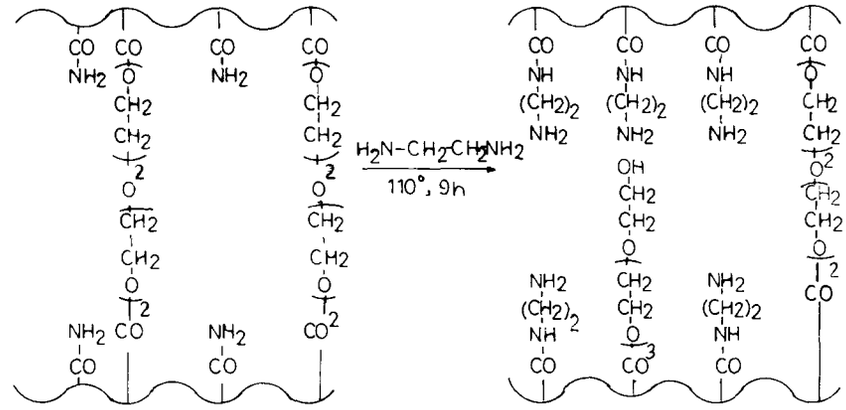
Figure 1 Variation in amino capacities with extent of crosslinking. (a) Poly(2N aminoethyl acrylamide)s and (b) poly(2N aminohexyl acrylamide)s

capacity increases first with the increase of crosslink density and then decreases with further increase in crosslinking. This appears to be due to the increased restriction imposed on the molecular mobility of the chain segments by the closer molecular packing in a tightly crosslinked system. However, the amino capacities of the DVB-crosslinked poly(amino acrylamide)s are lower than those of NNMBA-crosslinked copolymers due to the hydrophobic and rigid nature of DVB. In NNMBA-crosslinked poly(aminohexyl acrylamide)s, amino capacities are very low; they first increase and then decrease sharply with increase of crosslink density. In DVB-crosslinked poly(aminohexyl acrylamide)s, amino capacity increases first and then remains at the same value, showing that increased crosslinking does not have much effect on amino capacity. Amino capacities of poly(aminohexyl acrylamide)s are far below those of poly(aminoethyl acrylamide)s. There are chances of multiamidation at either end of the diamine, leading to additional crosslinking in the system[25] (*Scheme 3*). This additional crosslinking reduces the number of free amino

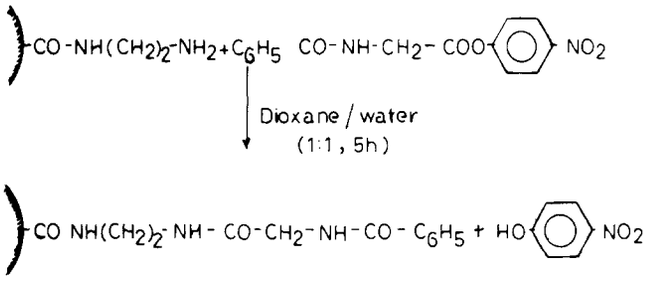


Scheme 3 Multiamidation of diamine at either end

groups and gives a lower value of amino capacity. The amidation of polar TEGDA-crosslinked poly(acrylamide)s presents interesting results. The amino resins derived from these copolymers show a regular increase in amino capacities with increase in crosslink densities. TEGDMA-crosslinked poly(aminoethyl acrylamide)s show a sharp decrease first and then a steady increase with increase of crosslink density. This anomalous behaviour can be explained as follows. During the basic conditions of amidation, some of the diester linkages of the oligooxy-



Scheme 4 Possible transamidation of the diester linkages



Scheme 5 Aminolysis of *N*-benzoylglycine 4-nitrophenyl ester by the polymeric amines

ethylene crosslinking agent in the tightly crosslinked matrix undergo hydrolysis to free acid groups, which then undergo transamidation giving an increased number of free amino groups (*Scheme 4*). Such a phenomenon has been observed by Egawa *et al.*[26].

Aminolysis of active ester by polymeric amines

In order to delineate the reactivities of the amino functions in the polymeric amines towards peptide bond formation, a model reaction was carried out. The reaction resembles the peptide bond formation in peptide synthesis[27]. *N*-Benzoylglycine 4-nitrophenyl ester was treated with the amino resins in a dioxane/water mixture[28] (*Scheme 5*). The extent of peptide bond formation was estimated. *Figure 2* represents the dependence of functional group reactivity on molecular character. It was found that reactivity of the amino functions in the amino resins towards peptide bond formation was definitely influenced by the nature and extent of crosslinking. Hydrophobic DVB-crosslinked poly(aminoethyl acrylamide)s showed the maximum coupling efficiency.

As the crosslink density increased from 5 to 10 mol% there was a drop in percentage coupling, but it soon began to rise to a maximum value of 94% when the crosslink density reached 20%. In the relatively hydrophilic TEGDA and TEGDMA-crosslinked poly(aminoethyl acrylamide)s, the shapes of the aminolysis curves are identical. Coupling efficiency first decreases up to 10–15% crosslinking and then increases sharply at 20% crosslinking. This increased reactivity of these amino resins is due to the increased availability of amino groups

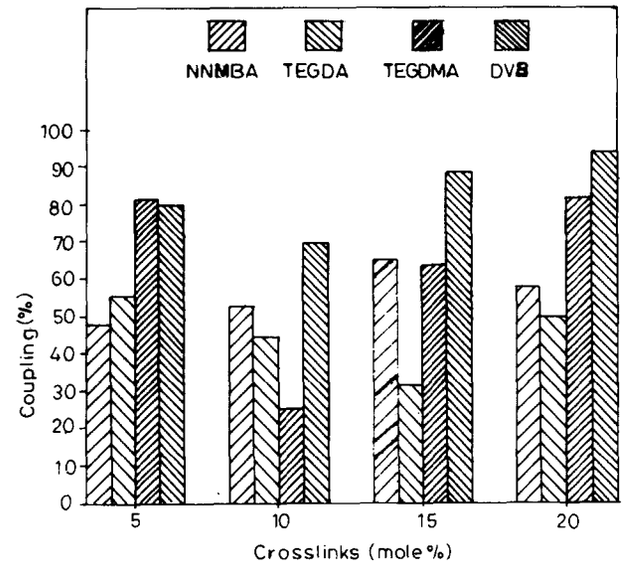


Figure 2 Dependence of functional group reactivity on molecular character

in a highly hydrophilic matrix with 20% flexible crosslinks. Coupling efficiency in NNMBA-crosslinked poly(aminoethyl acrylamide)s first increases steadily up to 15% crosslinks and then decreases with increasing crosslink density.

These observations suggest that for effective peptide bond formation, the resin support should have a proper blend of hydrophilic and hydrophobic character. Hydrophilic supports show decreased reactivity while hydrophobicity induced by DVB crosslinks in acrylamide–DVB resins offers excellent results in peptide coupling. The hydrophilic–hydrophobic balance of the support can be adjusted by varying the nature and extent of crosslinking. Thus the reactivity of functional groups is dependent on the variables of polymer synthesis[3]. These resins can be easily prepared and functionalized to low amino capacities. They possess the hydrophilic–hydrophobic balance essential for smooth and straightforward reaction. Hence they offer favourable conditions for peptide synthesis. The amino resins with a longer spacer arm of six methylene groups ($-NH-(CH_2)_6-NH_2$) showed decreased reactivity.

Morphological studies

Polymer morphology influences its reactivity[3,29,30]. Examination of the surface characteristics of the co-polymers by scanning electron microscopy reveals the presence of extensive channels and pores on the surface (*Figure 3*). DVB-crosslinked polyacrylamide possesses a highly ordered structure with a regular surface feature. This is probably due to the rigid and compact structure of DVB. The higher the extent of crosslinking, the greater the rigidity and order in chain packing. This probably results in the increased reactivities of the acrylamide–DVB resins. The amidated resins are more porous due to the presence of the hydrophilic aminoethyl groups. High porosity of the resin offers certain advantages: it permits good flow properties and does not hinder permeation by high molecular weight reagents[3].

Thermal behaviour

The thermal behaviour of certain acrylamide copolymers has been investigated by Vilcu *et al.*[31]. A proper understanding of the thermal stability of a crosslinked polymer is essential for predicting its suitability in various synthetic manipulations. The thermal behaviour of crosslinked poly(acrylamide)s is illustrated in *Figure 4*. All the copolymers are thermally stable up to 600°C. The first endotherm near 100°C in all four thermograms reveals the expulsion of moisture from the samples. At 300°C the second stage of decomposition takes place. This is due to the expulsion of water molecules physically bound to the hydrophilic polymer by hydrogen bonding and van der Waal's forces of attraction (free water or mobile water or freezing water)[32]. The endotherm at 350°C denotes the expulsion of water molecules chemically bound to the polymer (non-freezing water or bound water). The exotherm at around 500°C is characteristic of the thermal decomposition of the polymers. The d.t.a. curves of crosslinked poly(acrylamide)s suggest that they are thermally stable under a wide range of temperatures.

Swelling characteristics

The swelling characteristics of the 10% crosslinked polymers in solvents usually employed for peptide synthesis are shown in *Figure 5*. Maximum swelling of the resins was observed in highly polar solvents such as dimethylformamide and dioxane. *N*-Methyl-2-pyrrolidone also showed fairly good swelling. This is due to the compatibility of the resins with the solvents. The solvent compatibility of resins can be adjusted by varying the mole ratio between the monomer and the crosslinker in the copolymer. The polar, hydrophilic crosslinking agents such as TEGDA and TEGDMA induce polarity and hydrophilicity on the acrylamide copolymer; under the most favourable swelling medium, the pores and channels become completely filled by the solvents, leading to an effective swelling of the resin. Further, the swelling characteristics of a network are greatly influenced by

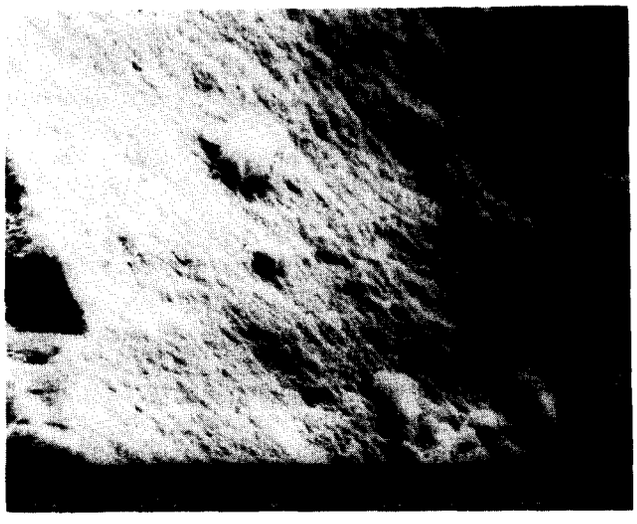


Figure 3 Scanning electron micrograph of DVB-crosslinked poly(acrylamide)

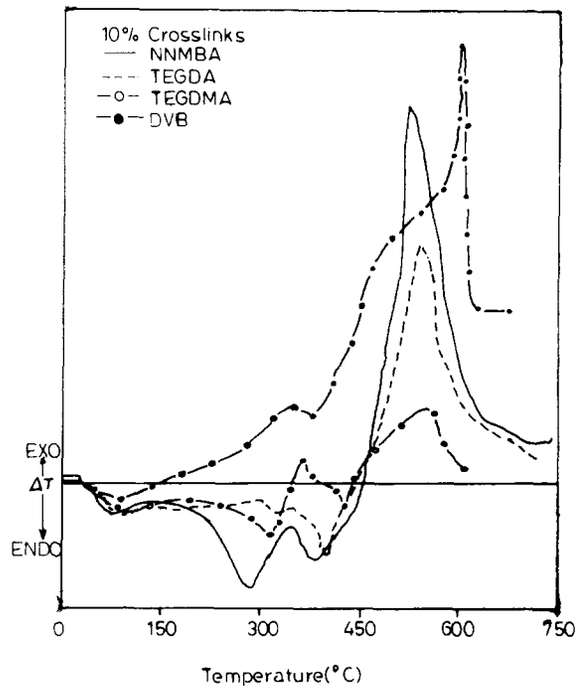


Figure 4 Thermal behaviour of crosslinked poly(acrylamide)s

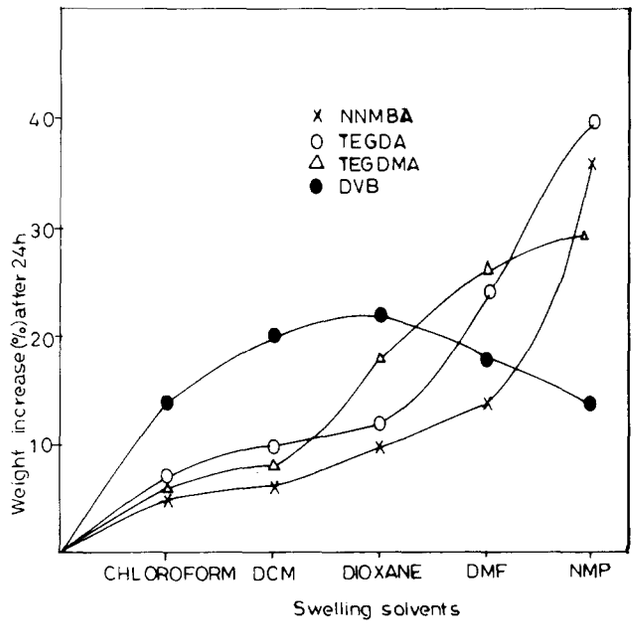


Figure 5 Swelling profiles of 10% crosslinked poly(acrylamide)s

the conditions under which the network has been formed[33]. De Boer and Pennings[34] observed that a solution-polymerized network could swell to a greater extent than bulk-polymerized samples. This is due to the macromolecular chains assuming the most probable extended conformations when polymerized in solution. Consequently, in the deswollen or dehydrated state, the average end-to-end distances will be shorter than in the 'uncrosslinked state'[35]. Such networks in the dry state would possess an extreme tendency to become solvated. Po *et al.*[36,37] refer to such networks as 'high free-energy networks' or 'hungry networks'. The nature of the solvent exerts a marked influence in the aminolysis reaction. Morawetz observed that the relative rates of aminolysis of polymer-bound active esters were considerably influenced by the solvent medium[38]. Devaky *et al.* observed that aminolysis of 4-nitrophenyl acetate by poly(2N aminoethyl acrylamide)s is gradually affected by the composition of the solvent mixture (dioxane/water) employed for aminolysis[18]. These observations point to the relevance of the local effective medium in the vicinity of the polymer. Effective permeation of the resin matrix occurs under the influence of a 'good' solvent, leading to the exposition of the reactive amino functions for peptide bond formation. The swellability of these resins in various polar and apolar solvents is a measure of the hydrophilic–hydrophobic balance, which in turn is dependent on the fine molecular character of the resin.

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