Synthesis, Characterization, and Application of Butanediol Dimethacrylate Cross-Linked Polystyrene: A Flexible Support for Gel Phase Peptide Synthesis

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ABSTRACT: We have developed a new cross-linked polymeric support by the copolymerization of styrene and butanediol dimethacrylate (BDODMA) for the solid-phase synthesis of peptides. The resin support was synthesized in various cross-linking densities by radical aqueous suspension polymerization. The copolymer was characterized by IR and ^{13}C NMR techniques. The shape, size, and morphological features of the cross-linked polymer bead were analyzed by scanning electron microscopy. PS-BDODMA polymer support showed excellent swelling properties in all types of solvents that are used in solid-phase peptide synthesis (SPPS). The stability of polymer in various reagents and solvents was tested by IR. The polymer does not show any change in its IR spectrum even after 48 h treatment with TFA, 20% piperidine in DMF, aqueous NaOH, hydroxylamine in aqueous MeOH, and liquor ammonia. The resin can be functionalized with chloromethyl, aminomethyl, and hydroxymethyl groups under various reaction conditions. The efficiency of this new support was demonstrated by the synthesis of few model peptides with very high purity and yield. These peptides were characterized by MALDI TOF MS and amino acid analysis.

Introduction

The classical solid-phase stepwise synthesis of peptide was introduced by Bruce Merrifield, describing the synthesis of a simple tetrapeptide using a PS-DVB resin.[1] This invention formed the basis of a new technique in organic chemistry, and it has been widely used until now with some refinements. This approach has been improved and generalized to the synthesis of complicated peptides including small proteins, long oligonucleotides, and small organic molecules.[2−4] The success of this technique often depends on the mechanical stability, compatibility, and swellability of the resin with a range of hydrophilic and/or hydrophobic solvents. The mechanical stability of the support was important since the resin was repeatedly treated with various types of reagents and solvents during the synthesis. Physicochemical incompatibility of the growing peptide chain and the insoluble polymer network can affect the purity of the target peptide. A macromolecular polymer support that swells in both polar and nonpolar solvents can overcome most of these problems. Synthesis of medium-to-large peptide with high purity and homogeneity is still a challenging problem in PS-DVB resin, primarily because of its rigidity and hydrophobicity.[5] In this support the rate of attachment of a particular amino acid has been found to decrease with increase in peptide chain length. Another main reason for the poor yield and low purity of the peptide is the physicochemical incompatibility between the strong hydrophobic macromolecular environment with the growing peptide chain and the formation of unfavorable conformational characteristics of the resin bound peptide/or protein sequences.[6] To overcome the difficulties associated with PS-DVB resin, a series of new resins such as poly(ethylene glycol) polystyrene (PEG-PS), tentagel graft resin, tetraethylene glycol diacrylate cross-linked polystyrene, and hexanediol diacrylate cross-linked polystyrene resin were developed.[7−10] Other type of supports developed and tested over these years for SPPS include polyamides, cotton and other carbohydrates, poly(ethylene glycol)−poly(acrylamide) (PEGA), and cross-linked ethoxylate acrylate resin (CLEAR).[11−14]

An efficient support for SPPS was developed by introducing hydrophilic BDODMA cross-linker to polystyrene network. The support was prepared by radical aqueous suspension polymerization of styrene with BDODMA. The optimum hydrophilic−hydrophobic balance of PS-BDODMA resin helps it become more physicochemically compatible support with polypeptide and/or protein chain than the other styrene-based supports. The present paper discusses the synthesis, functionalization, characterization, and swelling properties of PS-BDODMA resin. Here we also discuss the efficiency of the new resin as a solid support for the syntheses of several challenging peptides.

Experimental Section

Materials. Styrene, 1,4-butanediol dimethacrylate (BDODMA), poly(vinyl alcohol) (PVA, MW ∼ 75 000), 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid, and cesium carbonate were purchased from Aldrich Chemical Co., USA. 4-(Dimethylamino)pyridine (DMAP), piperidine, dicyclohexylcarbodiimide (DCC), 2-(1*H*-benzotriazol-1-yl)1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU), Fmoc-amino acids, and Boc-amino acids were purchased from Novabiochem Ltd., UK. Thioanisol, ethanedithiol, and diisopropylethylamine (DIEA) were purchased from Sigma Chemicals Company, USA. Chloromethyl methyl ether (CMME) was prepared using literature procedure.[15] All solvents used were of HPLC grade purchased from E. Merck (India), BDH (India), and SISCO Chemicals (Bombay). IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The ^{13}C CP-MAS solid-state NMR measurements were conducted on a Bruker 300 MSL

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CP-MAS instrument operating at 75.47 MHz. The spectra were run with fine powder of polymer beads at room temperature, and a KelF rotor was employed for MAS. The samples were rotated with a spectral width of 25 000 Hz, the CP time was 22 min, and the number of scans was in the range 200−300. Each sample was rotated with two different spin rates, and by comparing the resultant spectra the spinning sidebands were eliminated. HPLC was done on a Pharmacia Akta purifier instrument using C-18 reverse phase semi prep. HPLC column. The amino acid analysis was carried out on an LKB 4151 Alpha plus amino acid analyzer. For this the peptide was hydrolyzed using 6 N HCl in a Pyrex glass tube fused under nitrogen for 15 h at 130 °C. Mass spectra of peptides were obtained with a Kratos PC-Kompact MALDI TOF MS instrument.

Butanediol Dimethacrylate Cross-Linked Polystyrene Copolymer. Inhibitors are removed from styrene and BDODMA by washing with 1% NaOH solution and distilled water and drying over calcium chloride. A four-necked reaction vessel equipped with a thermostat, Teflon stirrer, water condenser, and nitrogen inlet and a dropping funnel were used. A net volume of 1% solution of poly(vinyl alcohol) (∼75 000) was prepared by dissolving PVA (1.1 g) in double distilled water (110 mL) and added to the reaction vessel. A mixture of styrene (11.4 mL), BDODMA (0.45 mL), and benzoyl peroxide (0.5 g) dissolved in toluene (10 mL) was added to the reaction vessel by stirring the aqueous solution at 2000 rpm. A slow stream of nitrogen was bubbled in to the reaction mixture. The temperature of the reaction mixture was maintained at 80 °C using a thermostated oil bath, and the reaction was allowed to continue for 6 h. The copolymer obtained as beads of 100−200 mesh size was washed thoroughly with hot water (to remove the stabilizer), acetone (3 × 50 mL), benzene (3 × 50 mL), toluene (3 × 50 mL), and methanol (3 × 50 mL). The copolymer was further purified by Soxhlet extraction with DCM and MeOH and dried in a vacuum at 40 °C for 10 h to yield 8.5 g of dry beads (80%). IR (KBr): 1720 cm^{-1}, 1480 cm^{-1}, 755 cm^{-1}, 700 cm^{-1}. ^{13}C CP-MAS NMR: 145.30 ppm, 130.48 ppm, 67.47 ppm, 42.78 ppm.

Resin Functionalization. a. Chloromethyl Resin. PS-BDODMA resin (5 g) was swelled in DCM in a 100 mL flask for about 1 h, and excess DCM was filtered off. A mixture of chloromethyl methyl ether (30 mL), DCM (25 mL), and 1 M $ZnCl_2$ in THF (1 mL) was added to the swelled resin, and the suspension was refluxed at 45 °C for 3 h. The resin was filtered using a sintered glass funnel and washed with THF (3 × 50 mL), THF/water (1:1, 3 × 50 mL), THF/3 N HCl (1:1, 3 × 50 mL), THF (3 × 50 mL), and methanol (3 × 50 mL). Drying in a vacuum at 40 °C for 10 h yielded 5.163 g of chloromethyl resin. The resin was found to have a capacity of 0.68 mmol Cl/g as estimated by Volhard's method.[16] IR (KBr): 1720 cm^{-1}, 1480 cm^{-1}, 1420 cm^{-1}, 1258 cm^{-1}, 755 cm^{-1}, 700 cm^{-1}, 670 cm^{-1}. ^{13}C CP-MAS NMR: 145.30 ppm, 135.6 ppm, 130.48 ppm, 67.47 ppm, 48.30 ppm, 42.78 ppm.

b. Aminomethyl Resin. Chloromethylated PS-BDODMA resin (0.68 mmol Cl/g, 2 g) was swelled in NMP. After 2 h excess NMP was filtered off. Potassium phthalimide (1.26 g, 6.8 mmol) dissolved in NMP (20 mL) was added, and the reaction mixture was stirred at 120 °C for 12 h. The resin was filtered and washed with NMP (3 × 50 mL), dioxane (3 × 50 mL), ethanol (3 × 50 mL), and methanol (3 × 50 mL). The dried resin was suspended in ethanol (20 mL), and hydrazine hydrate (0.33 mL, 6.8 mmol) was added. The reaction mixture was refluxed for 8 h. The resin was collected by filtration and washed with hot ethanol (3 × 50 mL) and methanol (3 × 50 mL). The product resin was dried in a vacuum. The amino capacity of the resin = 0.67 mmol/g as estimated by the picric acid titration method.[17] IR (KBr): 1720 cm^{-1}, 1520 cm^{-1}, 1480 cm^{-1}, 755 cm^{-1}, 700 cm^{-1}.

c. Hydroxymethyl Resin. Chloromethylated PS-BDODMA resin (0.68 mmol Cl/g, 1 g) was suspended in methylcellosolve (20 mL), potassium acetate (0.67 g, 6.8 mmol) was added, and the reaction mixture was stirred at 130 °C. After 48 h the acetoxy resin formed was filtered and washed with NMP (3 × 50 mL), DCM (3 × 50 mL), ethanol (3 × 50 mL), and methanol (3 × 50 mL). The acetoxy resin was mixed with hydrazine hydrate (0.33 mL, 6.8 mmol) in NMP (6 mL) for about 72 h at room temperature, and the hydroxy resin was collected by filtration. The resin was washed with NMP (3 × 50 mL), DCM (3 × 50 mL), ethanol (3 × 50 mL), and methanol (3 × 50 mL) and dried in a vacuum. The resin (100 mg) was acetylated with an acetic anhydride−piperidine mixture (1:4, 3 mL). After 6 h distilled water (10 mL) was added and refluxed for 3 h; the mixture was cooled and filtered. Acetic acid formed was back-titrated with standard (0.1 N) NaOH. The estimated hydroxyl capacity of the resin was 0.67 mmol of OH/g. IR (KBr): 3400 cm^{-1}, 1720 cm^{-1}, 1480 cm^{-1}, 755 cm^{-1}, 700 cm^{-1}.

Attachment of C-Terminal Boc-Ala to the Resin. Boc-Ala (0.105 g, 0.34 mmol) was dissolved in 4:1 ethanol−water (5 mL), and a 1 M solution of Cs_2CO_3 was added dropwise to the solution until the pH was reached 7.0. The solvent was removed by azeotropic distillation with benzene. The white powdery cesium salt of Boc-Ala obtained was dried under vacuum. To the preswelled chloromethyl resin (200 mg, 0.14 mmol) in NMP was added the cesium salt of Boc-Ala dissolved in dry NMP (1 mL), and the mixture was kept at 50 °C for 48 h with occasional shaking. The resin was filtered and washed with NMP (3 × 50 mL), NMP:water (1:1, 3 × 50 mL), DCM (3 × 50 mL), MeOH (3 × 50 mL), and ether (3 × 50 mL) and dried in a vacuum. The resin was found to contain 0.66 mmol of Ala/g as estimated by the picric acid method.[17]

4-(4-Hydroxymethyl-3-methoxyphenoxy)butylamidomethyl PS-BDODMA Resin (HMPB-resin). Aminomethyl PS-BDODMA resin (1 g, 0.67 mmol/g) was swelled in DMF. After 1 h the resin was washed with 10% DIEA in DCM (5 × 50 mL), DCM (5 × 50 mL), and DMF (5 × 50 mL). 4-(4-Hydroxymethyl-3-methoxyphenoxy)butyric acid (2.3 mmol, 0.55 g), HOBt (2.3 mmol, 0.31 g), HBTU (2.3 mmol, 0.87 g), and DIEA (0.67 mmol, 0.086 g) mixture in DMF (3 mL) was added to the swelled resin, and the mixture was shaken well for 1 h. The resin was filtered and washed with DMF (3 × 10 mL) and diethyl ether (3 × 10 mL) and dried in a vacuum. The resin was negative to the sensitive Kaiser test showing the 100% attachment of the anchoring group.[18] The resin was found to have a hydroxyl capacity of 0.58 mmol/g.

Attachment of C-Terminal Fmoc-Amino Acid to the HMPB Resin. Fmoc-amino acid (10 mmol excess) was dissolved in DCM (10 mL). DCC (5 mmol excess) in DCM (10 mL) was added to the mixture and stirred for 1 h. The anhydride formed was filtered, and the solvent was removed from the filtrate under vacuum and dried. HMPB resin (200 mg, 0.12 mmol) was swelled in DMF (50 mL) for 1 h. Excess DMF was filtered off; Fmoc-amino acid anhydride and DMAP (0.014 g, 0.12 mmol) were dissolved in DMF (1 mL) and added to the resin, and the mixture was shaken for 1 h. The resin was filtered and washed with DMF (3 × 50 mL), isoamyl alcohol (3 × 50 mL), acetic acid (3 × 50 mL), and ether (3 × 50 mL) and dried under vacuum. The extent of attachment of amino acid was estimated by adding a 20% piperidine in DMF (3 mL) to a definite amount of resin. After 20 min the optical density (OD) of the solution was measured at 290 nm. From the OD value the amino capacity of the resin was calculated. The capacities of the resins are 0.55 mmol of Ala/g and 0.56 mmol of Gly/g.

Swelling Studies. The solvent absorption of various resins was determined by a centrifuge method. The resin (1 g) was placed in a glass sintered stick (G3) and the latter immersed in the solvent for 48 h. The stick was then transferred to a centrifuge tube, and the excess solvent was removed by centrifuging for 15 min. The stick and the contents were then weighed. A similar blank experiment was performed using an empty sintered stick. The data were expressed as the volume of the solvent absorbed by unit weight of dry resin (mL/g). In another experiment the volume occupied by unit weight of dry resin in its solvent swollen state (mL/g) was measured by noting the volume resulting when a definite weight of dry resin was added to a known volume of solvent in a small measuring cylinder.

Stability Studies. The stability studies of the resin were carried out in different reagents such as 100% TFA (10 mL),

20% piperidine in DMF (10 mL), 2 M aqueous NaOH (10 mL), 2 M NH_2OH in aqueous methanol (10 mL), and liquor ammonia (10 mL). The resin samples (100 mg of each) were separately stirred with the above-mentioned reagents. After 48 h the resin samples were filtered, washed thoroughly with ethanol (3 × 50 mL), water (3 × 50 mL), acetone (3 × 50 mL), DCM (3 × 50 mL), dioxane (3 × 50 mL), and ether (3 × 50 mL), dried, and weighed (100 mg); IR (KBr) spectra of these resins were compared with the original.

General Procedure for Peptide Synthesis. Peptide 1 was synthesized manually in a specially designed silanized glass vessel clamped to a mechanical shaker. Boc-amino acids were coupled to the resin by the DCC/HOBt coupling procedure. In a typical coupling procedure Boc-amino acid (2.5 equiv) was dissolved in NMP (1 mL), the solution was cooled to 0 °C, DCC (2.5 equiv) and HOBt (2.5 equiv) were mixed, and the mixture was stirred for 20 min at 0 °C. The white precipitate of dicyclohexylurea (DCU) formed was removed by filtration. The resin containing the C-terminal amino acid was swelled in NMP (50 mL). After 1 h excess NMP was filtered off, HOBt active ester solution of the next residue was added, and the mixture was shaken for 50 min. In certain sequences the coupling reaction was performed twice or thrice to achieve quantitative conversion. The coupling reaction was monitored by Kaiser's semiquantitative ninhydrin test. After coupling reaction the Boc group was removed by adding 30% TFA in DCM for 30 min, and the neutralization of the salt formed was carried out by adding 5% DIEA in DCM for 10 min. After each coupling, Boc deprotection, and neutralization steps, the resin was washed with DCM (5 × 50 mL) and NMP (5 × 50 mL). After the synthesis Boc protection of the N-terminal amino acid was removed with 30% TFA in DCM, and the resulting peptidyl resin was washed with DCM (5 × 50 mL), methanol (5 × 50 mL), and ether (5 × 50 mL) and dried in a vacuum.

Peptides 2 and 3 were synthesized using Fmoc-amino acids. All the Fmoc-amino acids were coupled to the C-terminal amino acid attached resin (1 equiv) by the HOBt active ester method. In a typical coupling step HOBt (2.5 equiv) and DCC (2.5 equiv) were added to Fmoc-amino acid (2.5 equiv) dissolved in DMF (1 mL). The mixture was stirred for 20 min, and the precipitated DCU was filtered off. The HOBt active ester was added to DMF swelled resin, and the coupling reaction was continued for 50 min. The extent of coupling was monitored by the Kaiser test.[18] Fmoc protection was removed using 20% piperidine in DMF. After each coupling and Fmoc deprotection steps the resin was washed with DMF (5 × 50 mL). After the synthesis the peptidyl resin was washed with DMF (5 × 50 mL), MeOH (5 × 50 mL), and ether (5 × 50 mL) and dried under vacuum.

Removal of Peptide from the Polymer Support. The peptidyl resin was suspended in a mixture of TFA (2.55 mL), thioanisol (150 μL), ethanedithiol (75 μL), phenol (75 μL), and double distilled water (150 μL). The mixture was kept at room temperature for 12 h in the case of peptide 1 and 4 h for peptides 2 and 3. The polymeric material was filtered off and washed with fresh TFA and rinsed with 10 mL of DCM. The filtrate was vacuum-evaporated at 40 °C to obtain a thick oily residue. The peptide was precipitated as white powder by adding ice-cold ether. The precipitate was washed thoroughly with cold ether (5 × 10 mL) to remove all the scavengers. The peptide was dissolved in glacial acetic acid and reprecipitated by adding cold ether. The precipitate was again washed with cold ether (5 × 10 mL). The peptide was further purified by dissolving in a 5% $HOAc/H_2O$ mixture and eluting through a Sephadex G-25 column. The eluting fractions containing the peptide were collected and lyophilized. The HPLC profile of each peptide shows only one major peak. The fraction corresponding to the major peak was collected and lyophilized. Amino acid analysis of these fractions gave the following results.

*Peptide 1.*Yield of crude peptide = 0.123 g (99.1%, based on the C-terminal Ala incorporated to the resin). Amino acid analysis: Ala, 1.00 (1); Ile, 0.98 (1); Leu, 1.09 (1); Gly, 3.16 (3); Val, 2.89 (3); Met, 0.79 (1). MALDI TOF MS: *m/z* 916.2 [$(M + H)^+$, 67%], 937.6 [$(M + Na)^+$, 100%], 955.9 [$(M + K)^+$, 55%], $C_{41}H_{74}N_{10}O_{11}S$, requires M^+ 915.158.

Peptide 2. Yield of crude peptide = 0.232 g (98.7%, based on the C-terminal Ala incorporated to the resin). Amino acid analysis: Ala, 1.00 (1); Ile, 2.10 (2); Thr, 0.81 (1); Arg, 0.98 (1); Met, 0.91 (1); Gly, 3.01 (3); Val, 4.24 (4); Lys, 4.88 (5); Asp, 0.91 (1). MALDI TOF MS: *m/z* 2027.6 [$(M + H)^+$, 100%], $C_{90}H_{168}N_{28}O_{22}S$, requires M^+ 2026.582.

Peptide 3. Yield of crude peptide = 0.145 g (99.1%, based on the C-terminal Gly incorporated to the resin). Amino acid analysis: Ala, 2.00 (2); Thr, 0.83 (1); Gly, 2.11 (2); Ile, 3.13 (3); Asp, 0.91 (1); Cys, 0.79 (1); Lys, 1.89 (2). MALDI TOF MS: *m/z* 1261.512 [$(M + H)^+$, 100%], $C_{53}H_{98}N_{15}O_{17}S$, requires M^+ 1260.512.

Synthesis of Acyl Carrier Protein Fragment (65−74) on Sheppard, PS-DVB, and PS-BDODMA Resins. PS-BDODMA-HMPB-Gly-Fmoc resin (200 mg, 0.11 mmol Gly), PS-DVB−HMPB-Gly-Fmoc resin (190 mg, 0.11 mmol Gly), and Sheppard-HMPB-Gly-Fmoc resin (210 mg, 0.11 mmol Gly) were used for the synthesis. The resins were taken in a manual peptide synthesizer and swelled in DMF (50 mL) for 30 min. The Fmoc protection was removed by treating the resins with 20% piperidine/DMF (10 mL) solution for 20 min. The resin was then washed thoroughly with DMF (6 × 20 mL). The respective amino acids required for the three resins were taken in 0.99 mmol quantity and mixed with HBTU (0.99 mmol) and HOBt (0.99 mmol) in a test tube and dissolved in 3 mL of DMF. DIEA (0.33 mmol) was added to the solution and mixed thoroughly. One milliliter of the solution was added to each of the manual synthesizers containing the respective resins and kept for 40 min with occasional shaking. The solution was filtered off, and the resins were washed with DMF (6 × 20 mL). After attaching the remaining amino acids of the acyl carrier fragment (66−74), Fmoc protection of the N-terminal amino acid was removed with 20% piperidine solution in DMF. The resins were washed with DMF (6 × 20 mL), NMP (5 × 20 mL), DCM (5 × 20 mL), MeOH (5 × 20 mL), and ether (5 × 20 mL) and dried under vacuum.

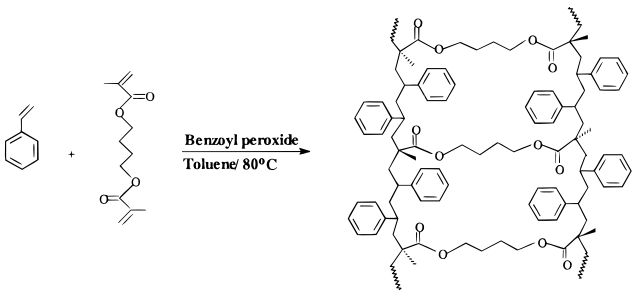
The peptidyl resins were suspended in a mixture of TFA (2.85 mL), water (75 μL), and ethanedithiol (75 μL), and the mixture was kept at room temperature. After 4 h the suspension was filtered and washed with TFA (1 mL), and the filtrate was concentrated under reduced pressure. The peptide was precipitated by adding ice-cold diethyl ether, washed with ether (6 × 10 mL), and dried.

Results and Discussion

The important parameters that determine the physicochemical properties that render a polymer support favorable for peptide synthesis are the chemical nature and topographical structure of the polymer matrix. The topology of the polymer matrix is highly influenced by the chemical nature of the monomers and mole percentage of cross-linking agent. The chemical nature and mole percentage of cross-linking agent provide the desired mechanical integrity and polarity of the resins. PS-BDODMA support shows comparable mechanical stability with PS-DVB resin, and the hydrophilic nature of the cross-linker helps the resin to be physicochemically compatible with the resin bound peptide.

In the initial phase of this work bulk polymerization of BDODMA and styrene was carried out. The polymer obtained after polymerization was grounded and sieved to particles of below 500 mesh size. But this process results in particle with irregular shape, and when the resin is functionalized with the chloromethyl group using CMME, the resin becomes more rigid and powdered. Suspension polymerization has been proved to be the most useful technique for synthesizing cross-linked polymer support principally because of the extremely convenient physical form of the beaded

Scheme 1. Synthesis of PS−BDODMA Polymer Support



product which lends itself to further conversions.[19] The polymer was synthesized with various cross-linking densities (1%, 2%, 3%, 4%, 6%, 8%, and 10%) by the free radical aqueous suspension polymerization using toluene as diluent and benzoyl peroxide as initiator (Scheme 1). The insoluble polymer support was obtained as spherical uniform beads. The beads were sieved, and the main fractions obtained were of 100−200 mesh size. The reproducible results were obtained in the preparation of beads of 100−200 mesh size by adjusting the amount of stabilizer PVA, geometry of the vessel and stirrer, and the stirring rate. Scanning electron microscopy (SEM) of the polymer shows that they are uniform spherical beads (Figure 1a). Functionalization of the resin results in some morphological changes on the resin (Figure 1b). Morphological changes are also observed in the SEM photograph of the peptidyl resin (Figure 1c). These changes are not the result of the pulverization of the bead, and it is the result of any problems during the filtration stages.

The PS-BDODMA resin was characterized by IR and ^{13}C CP-MAS NMR spectroscopy. The resin was powdered and pelletized with KBr. The IR spectrum of the resin shows a sharp band at 1720 cm^{-1} corresponding to the ester carbonyl of the cross-linker besides the usual peaks of polystyrene. The solid-state ^{13}C CP-MAS NMR spectrum of the resin (Figure 2a) shows an intense peak at 130.48 ppm which corresponds to aromatic polystyrene carbons and a small peak at 145.30 ppm for the C-3 carbon of styrene. The peak at 42.78 ppm corresponds to the backbone methylene carbon of the polymer. The methylene carbon of the cross-linking agent appears as a small peak at 67.47 ppm. The resin was functionalized with the chloromethyl group under various reaction conditions using chloromethyl methyl ether (CMME) and $ZnCl_2$/THF as catalyst. Chlorine capacity of the resin was determined by Volhard's method.[16] The reaction can be easily controlled, and the chloromethyl resin of the desired chlorine capacity can be prepared by adjusting the amount of reagent, amount of catalyst, temperature, and time duration of the reaction. The time dependence of chloromethylation of the resin at constant temperature and temperature dependence of chloromethylation of the resin at constant time are given in Figure 3a,b. The time-dependent chloromethylation reaction shows that the rate of functionalization was very high in the first 3 h, and the reaction rate is found almost steady with passage of time. The temperature-dependent chloromethylation reaction shows a high rate of functionalization up to 55 °C, but above this temperature the rate of functionalization reduces drastically. This may be due to the vaporization of chloromethyl methyl ether above 55 °C. The functionalized polymer is characterized by IR and ^{13}C CP-MAS NMR spectroscopy (Figure 2b). The IR (KBr) spectrum shows a band at 670 and 1420 cm^{-1} for C−Cl stretching and 1250 cm^{-1} for H−C−Cl vibration. The ^{13}C CP-MAS NMR spectrum shows a peak at 48.30 ppm for methylene carbon of the chloromethyl group and a small peak at 135.6 ppm for C-6 carbon of the polystyrene ring.

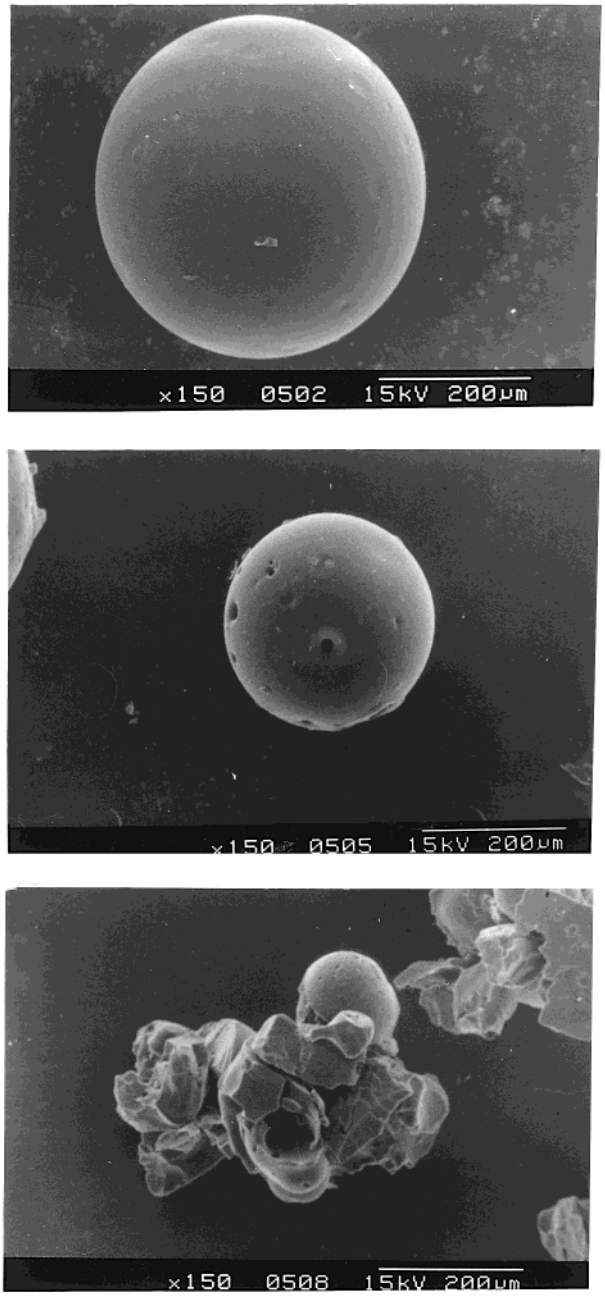


Figure 1. (a, top) Scanning electron micrograph showing the PS-BDODMA spherical bead prepared by suspension polymerization. (b, middle) Scanning electron micrograph showing the chloromethylated PS-BDODMA bead. Functionalization resulted in some morphological changes in the spherical bead. (c, bottom) Scanning electron micrograph showing the peptidyl resin bead. Peptide synthesis results in morphological changes to the resin. Working magnification of all the resins, 150×; accelerating voltage, 15.0 kV.

The aminomethyl resin was prepared from the chloromethyl resin by treating with potassium phthalimide followed by hydrazinolysis. The product gives a deep blue color with ninhydrin. The amino capacity of the resin was determined by the picric acid method.[17] The resin gives a characteristic IR (KBr) absorption at 1720

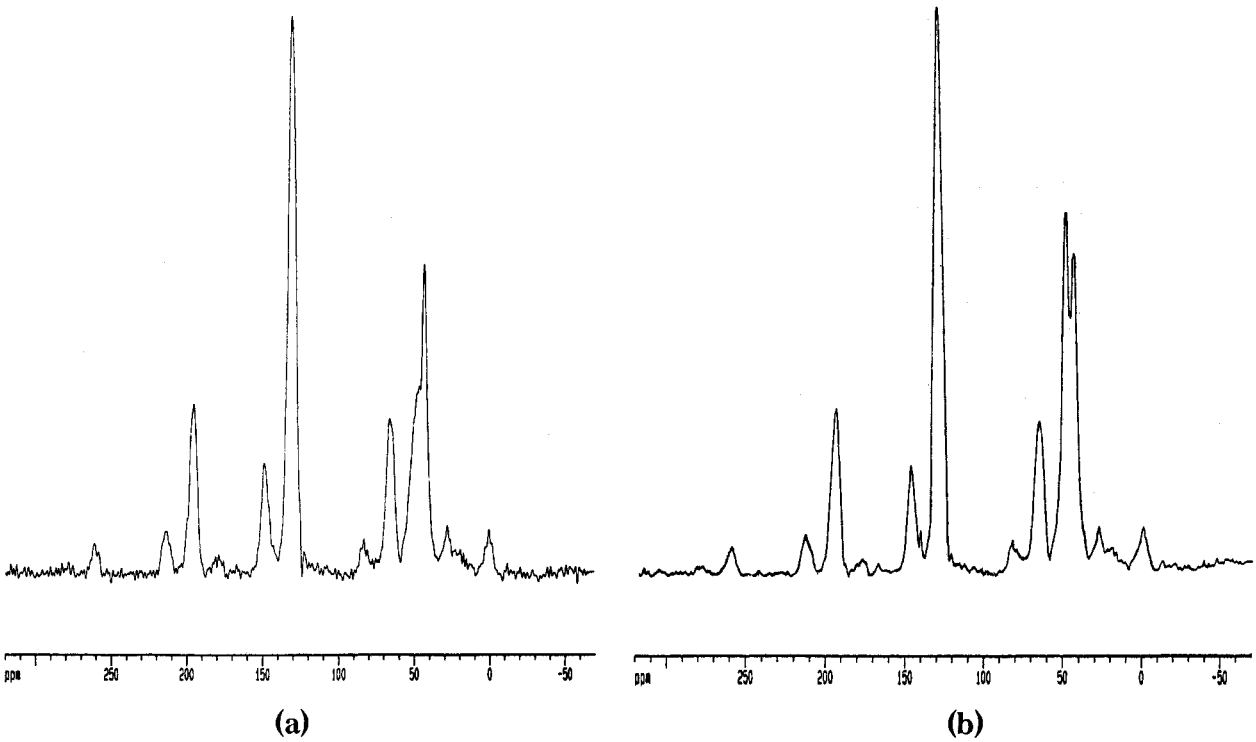


Figure 2. ^{13}C CP-MAS NMR spectrum of (a) PS-BDODMA support and (b) chloromethyl PS-BDODMA support.

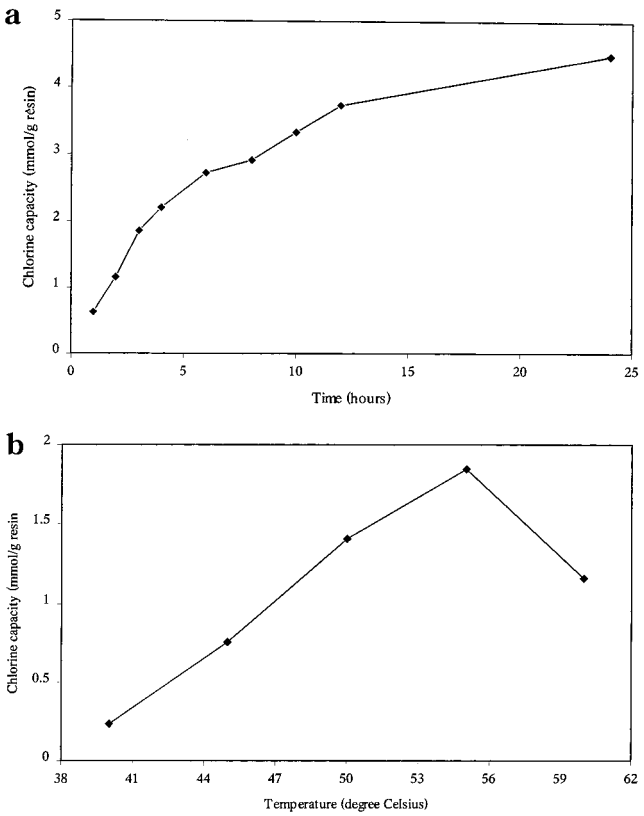


Figure 3. (a) Time-dependent chloromethylation of PS-BDODMA resin at 55 °C. (b) Temperature-dependent chloromethylation of PS-BDODMA resin.

cm^{-1}, 1480 cm^{-1} (ester), and 1520 cm^{-1} (amino). The hydroxymethyl group was introduced to the resin by treating the chloromethyl resin with potassium acetate followed by hydrazinolysis of the product. This support was then characterized by IR spectroscopy. The resin-KBr pellet shows a characteristic absorption at 1720 and 1490 cm^{-1} corresponding to the ester functional group of the cross-linker and an absorption at 3400 cm^{-1} corresponding to hydroxyl functional group.

Swelling and Stability Studies of Polymer Support. Swelling of polymer is one measurement of its solvation by a particular solvent. Since solvation is one of the necessary conditions for chemical reaction in gel phase, a useful parameter to determine the potential of the polymer as a support for peptide synthesis is its swelling properties in both polar and nonpolar solvents. For an effective SPPS, the accessibility of the growing resin bound peptide chain to reagent and solvents was very important. The reactive functional groups in the resin will have the maximum accessibility toward the reactants only when polymer matrix swells extensively in the solvating medium.[20] Chloromethyl PS-BDODMA also shows the same extent of swelling as the original PS-BDODMA resin, indicating that there is no additional cross-linking during chloromethylation. Swelling of 1% PS-DVB resin is compared with 2% PS-BDODMA and chloromethyl PS-BDODMA resin (Figure 4a). PS-BDODMA support swells in a wide range of polar and nonpolar solvents (Figure 4b). These characteristics of the support help the easy accessibility of functional groups on the polymer support to various reagents and solvents used in the synthesis. All these properties make the new resin a superior support compared to PS-DVB resin. The 1% PS-BDODMA resin was not used for the synthesis because it gets pulverized when the number of amino acid residues exceeds ten. This problem also results in difficulties when the reagents and solvents used for the synthesis were removed by filtration. PS-BDODMA resins with more than 4% cross-linking density are not used as support for SPPS. These resins are rigid, and their swelling characteristics decreases with increase in cross-linking densities. The 2%, 3%, and 4% PS-BDODMA resins are found to be good supports for the synthesis. Since the 2% cross-linked support shows better swelling charac-

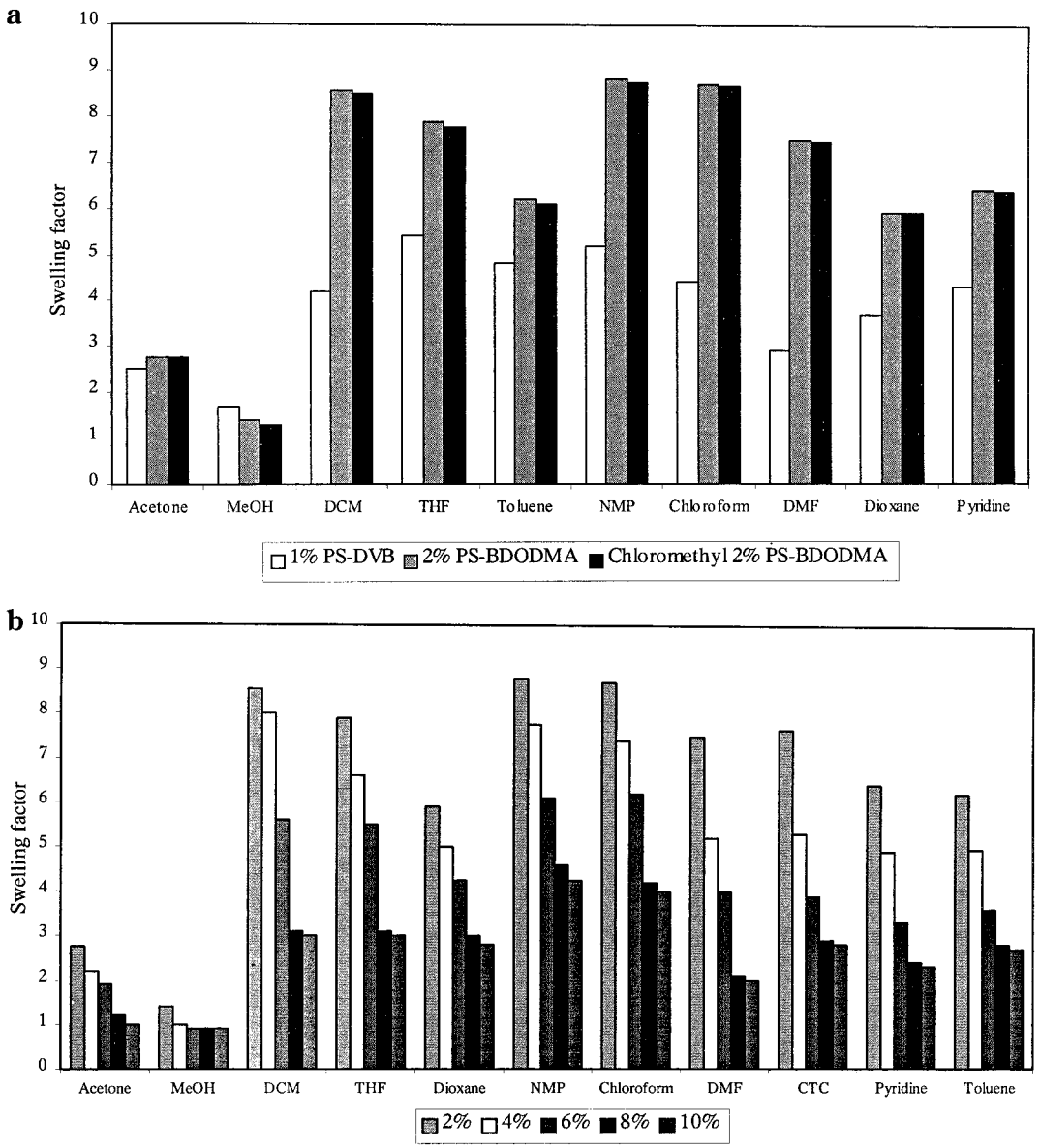


Figure 4. (a) Swelling comparison of 1% PS-DVB with 2% PS-BDODMA resin and chloromethyl 2% PS-BDODMA resin. (b) Swelling comparison of PS-BDODMA resin with various cross-linking densities in different solvents.

teristics compared to 3% and 4% cross-linked supports in different solvents, it was selected for the synthesis of all these peptides.

The capability of the resin as support for SPPS is also tested in a continuous flow method using a NovaSyn semiautomatic peptide synthesizer. Packing down of the 2% PS-BDODMA resin in pumped column system does not generate much back-pressure which prevents the free flow of reagents and solvents through the swollen resin beads. Back-pressure is not observed even after incorporating 15 amino acid residues to the resin. For the synthesis of peptides having more than 20 residues the resin beads were packed in to the column by mixing with small glass pieces in order to prevent the possibility of back-pressure.

Chemical Stability of PS-BDODMA Support. The chemical stability of the resin is one of the prime requirements of an efficient support for SPPS. The 2% PS-BDODMA resin was found to be stable even after vigorous conditions of functionalization. This support has comparable physical and mechanical properties as that of 2% PS-DVB support, permitting identical manipulations such as shaking and filtration when used as support for SPPS. The stability of the resin was tested in various conditions of peptide synthesis. A 48 h treatment of the resin with 33% TFA/DCM mixture which was used for the Boc removal does not show any change in the IR spectrum. Similarly, piperidine−DMF (1:4) treatment of the resin for 48 h, the reagent used for the Fmoc removal, shows no nitrogen incorporation as revealed by the IR spectrum. When the resin is treated with neat TFA for 48 h, no weight loss and no significant change in the IR spectrum were observed. The ester group present in the resin was stable enough to withstand the nucleophilic cleavage by bases stronger than piperidine. To investigate this, the PS-BDODMA support is incubated in 2 M aqueous NaOH, 2 M NH_2OH in aqueous MeOH, and liquor ammonia. After 48 h the IR spectrum of the resins shows no change, suggesting that the support resists the strong basic treatment even after 48 h. All these observations (Figure 5) reveal that the cross-links are stable enough to withstand the various reaction conditions employed in the SPPS.

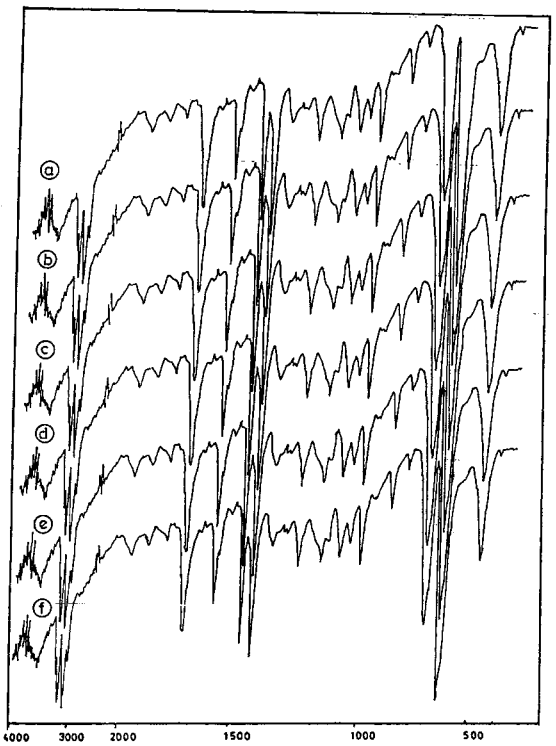


Figure 5. IR (KBr) spectra of PS-BDODMA: (a) original; after 48 h treatment of the resin with (b) neat TFA, (c) 20% piperidine-DMF, (d) 2 M aqueous NaOH, (e) 2 M NH_2OH in aqueous MeOH, and (f) liquor ammonia.

Comparative Synthesis of Acyl Carrier Protein Fragment (65−74) on PS-BDODMA, PS-DVB, and Sheppard Resins. PS-BDODMA, PS-DVB, and Sheppard resins attached with HMPB handle were used for the synthesis of acyl carrier protein fragment. The C-terminal Fmoc-glycine was attached to the resin via an ester bond using the preformed symmetric anhydride of Fmoc-glycine. The extent of attachment was measured from the UV absorbance of the adduct of dibenzofulvene and piperidine. All resins having the same capacity were used for the synthesis. After the synthesis the peptide was removed from the corresponding resins under the same cleavage conditions. The purity of the acyl carrier protein fragment from each resin was tested by the HPLC technique using a Sephasil Peptide C-18 column. HPLC profiles of crude peptides obtained from different resins are shown in Figure 6a−c. All eluting fractions corresponding to different peaks of each peptide obtained from the three resins were collected and analyzed by amino acid analysis and MALDI TOF MS. From the HPLC profile the peak area corresponding to acyl carrier protein fraction showed that PS-DVB resin yielded only about 38% of pure peptide, the Sheppard resin yielded about 67% of pure peptide, and PS-BDODMA yielded about 71% of pure peptide under the same synthetic conditions. The comparative analysis showed that the PS-BDODMA resin can be used as a better solid support for peptide synthesis than the PS-DVB resin and is as efficient as the Sheppard resin.

Manual SPPS. All peptides were synthesized manually on a 2% PS-BDODMA support in a shaking glass reaction vessel containing a sintered ware filter on one side and a receiving adaptor which can be fitted with calcium chloride guard tube on the other side. Peptide 1 was synthesized on a chloromethyl PS-BDODMA resin using Boc chemistry. The C-terminal Boc-amino acid was attached to the preswelled chloromethyl polymer support via an ester bond using the cesium salt method.[21] The extent of incorporation of amino acid was estimated by the picric acid method after the removal of Boc protection showed that it was 100%. This was also confirmed by the absence of any detectable amount of residual chlorine by Volhard's method. Boc deprotection was carried out with 30% TFA in DCM, and the neutralization was carried out with 5% DIEA in DCM. All coupling reactions were mediated by preformed HOBt active ester of the respective Boc-amino acid.

Peptide 1: Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala.

Peptide 2: Lys-Asn-Val-Gly-Lys-Lys-Val-Gly-Met-Lys-Val-Val-Arg-Thr-Gly-Ile-Lys-Ile-Ala.

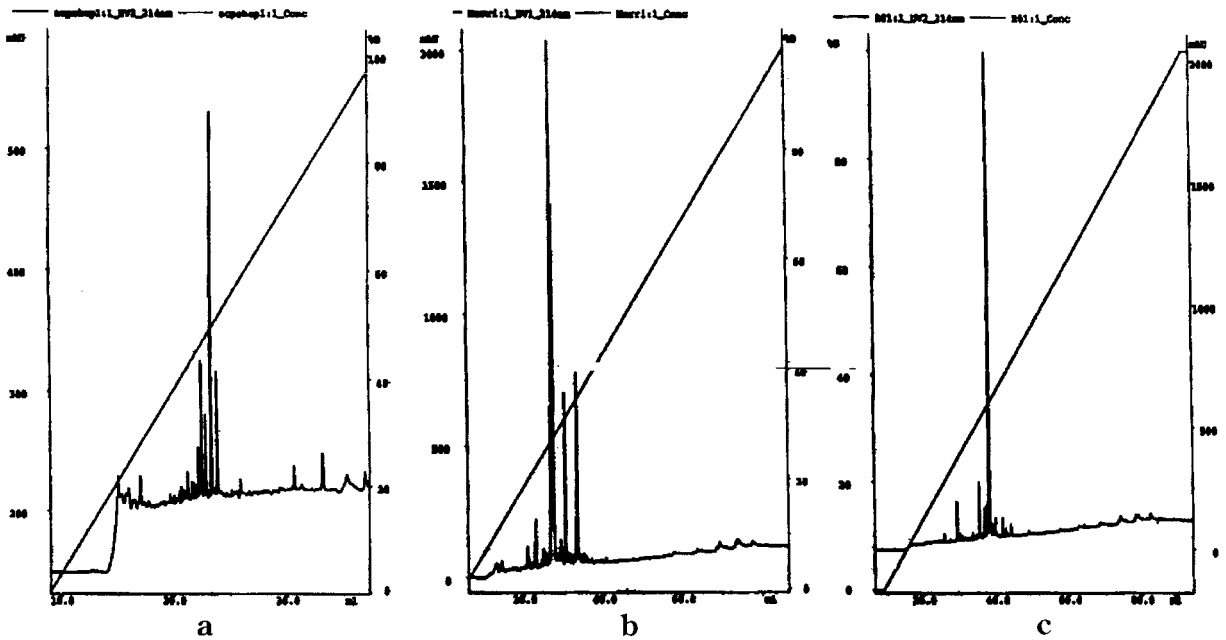


Figure 6. HPLC time-course analysis of peptide synthesized on (a) Sheppard resin, (b) PS-DVB resin, and (c) PS-BDODMA resin using the buffer (A) 0.5 mL of TFA in 100 mL of water and (B) 0.5 mL of TFA in 100 mL of acetonitrile:water (4:1). Flow rate: 0.5 mL/min. Gradient used: 0% B in 5 min, 100% B in 45 min, and 100% B in 50 min.

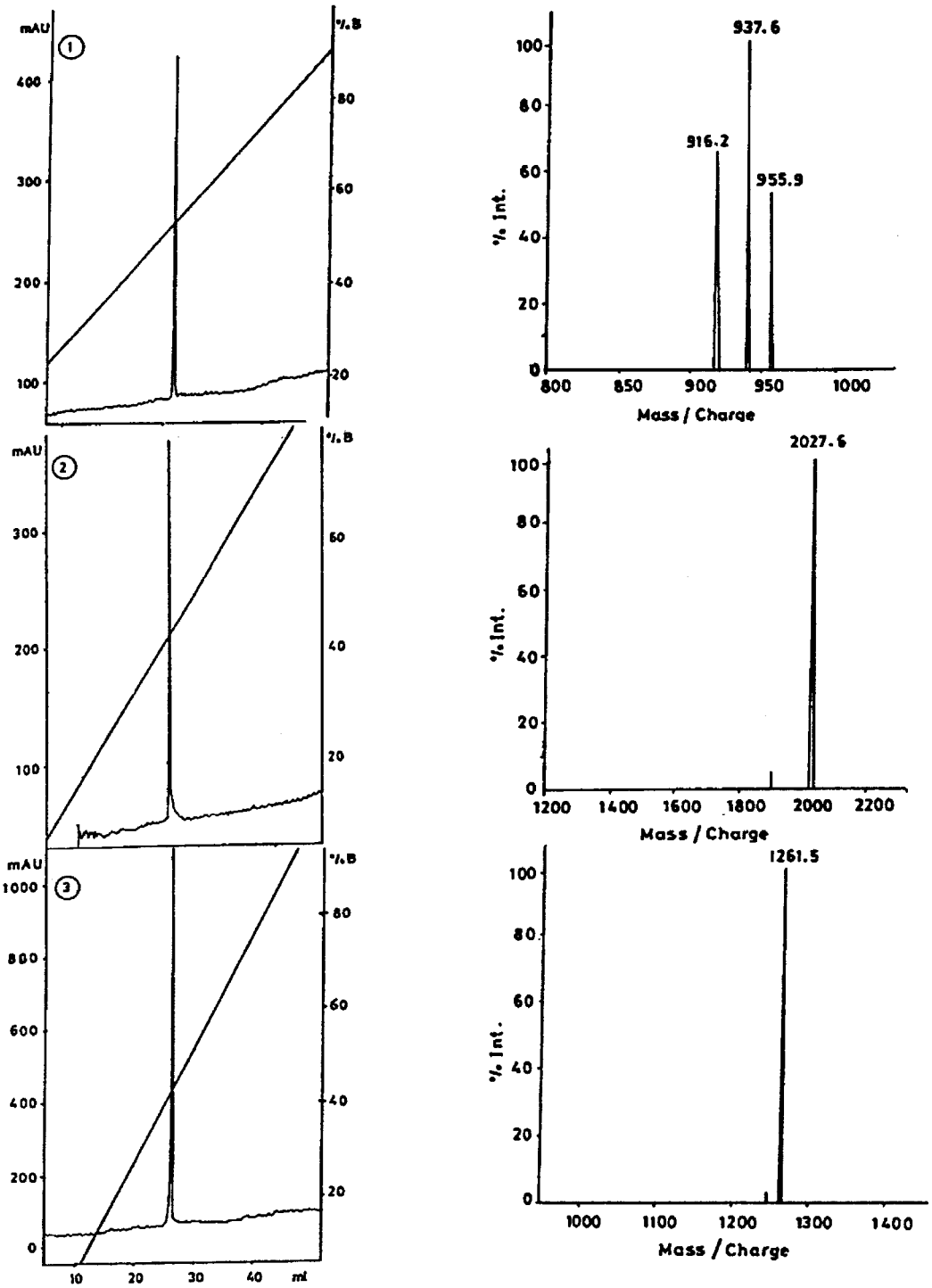


Figure 7. HPLC time-course analysis of peptides using the buffer (A) 0.5 mL of TFA in 100 mL of water and (B) 0.5 mL of TFA in 100 mL of acetonitrile:water (4:1). Flow rate: 0.5 mL/min. Gradient used: 0% B in 5 min, 100% B in 45 min, and 100% B in 50 min. (1) peptide 1; (2) peptide 2; (3) peptide 3; their corresponding MALDI TOF mass spectrum.

Peptide 3: Thr-Gly-Ile-Asp-Ile-Ala-Ala-Cys-Lys-Ile-Lys-Gly.

Peptide 2 and 3 were synthesized on a 4-(4-hydroxymethyl-3-methoxyphenoxy)butylamidomethyl PS-BDODMA resin using Fmoc-amino acids. The C-terminal Fmoc-amino acid was attached to the preswelled resin via an ester bond using preformed symmetric anhydride of Fmoc-amino acid in the presence of DMAP as catalyst. The contact between the activated Fmoc-amino acid derivative and DMAP was minimized to prevent the racemization and the cleavage of the Fmoc protecting group that can result in the formation of a dipeptide on the resin. Therefore, if the first attachment was not completed in 1 h, the reaction was repeated once again with fresh reagents. Deprotection of Fmoc group was performed with 20% piperidine in DMF, and the coupling reactions were mediated by HOBt/DCC. The HPLC profile (Figure 7a−c) and amino acid analysis of the data of these peptides showed that all acylation reactions of most of the amino acids were completed with single coupling in 50 min. But in some sequences a second coupling was performed to achieve 100% coupling. Side reactions such as the formation of diketopiperazine (DKP), though it was highly sequence de-

pendent, was not observed during the synthesis. However, coupling of the third amino acid was followed immediately after the deprotection of the second amino acid.

In the case of peptide 1 and peptide 3 the yield was more than 98% as indicated by the amino estimation of the remaining peptide bounded to the resin using the picric acid monitoring method. This was confirmed by the amino acid analysis and sequencing of the remaining resin bound peptide. The HPLC peak area corresponding to the target peptide in the crude sample also revealed that the yield was more than 98%. In the case of peptide 2 the major component in the crude cleavage product was isolated by preparative HPLC. Amino acid analysis and MALDI TOF MS result showed that the major peak obtained belong to the target peptide. The yield of peptide 2 was found to be more than 95% as calculated from the HPLC peak area of the target peptide in the crude sample. The small peak present in the HPLC profile may be due to some modification of the acid-sensitive amino acid present in the sequence.

Conclusions

PS-BDODMA resin was prepared by suspension polymerization using different mole percentages of BDODMA with styrene. It serves as a new class of polymeric support for solid-phase peptide synthesis. These supports are stable under all peptide synthetic conditions. The ester cross-linkage of the resin was stable enough to withstand strong acidic and alkaline conditions. The optimum hydrophobic−hydrophilic balance of the resin results in high swelling in different nonpolar and polar solvents, and therefore the range of chemistry that could be conducted on the support makes it an efficient support for different organic reactions. The ease of preparation, functionalization, and workup procedure are the advantages of the resin over conventional polymer supports. The physicochemical compatibility of the macromolecular support and the growing peptide chain help to synthesize peptides of very high purity and homogeneity. The enhanced coupling rate during peptide bond formation, high sensitivity in monitoring the coupling reactions, economical use of reagents, reactants, and solvents, and the yield and purity of the peptides are the advantages of the new PS-BDODMA resin support.

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