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Synthesis and characterization of glycerol dimethacrylate cross-linked polymethyl methacrylate: a resin for solid phase peptide synthesis

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Abstract

The new glycerol dimethacrylate cross-linked polymethyl methacrylate polymer support (GDMA-PMMA) for solid phase synthesis is presented. The synthesis of GDMA-PMMA resin is based on the cross-linking of GDMA with methyl methacrylate by free radical polymerization, affording a polymer containing ester and secondary hydroxyl groups. The polymer was prepared using benzoyl peroxide as initiator either via bulk polymerization or via suspension polymerization in polyvinyl alcohol, the latter yielding a beaded resin. The polymerization reaction was investigated with respect to the effect of amount of cross-linking agent in order to vary the swelling, loading and the mechanical stability of the resin. The polymer was characterized by FT-IR and ^{13}C CP MAS NMR spectroscopic techniques. The solvent uptake of the polymer was studied in relation to cross-linking and compared with Merrifield resin. The stability of the resin was tested in various synthetic conditions used in solid phase peptide synthesis. The resin was derivatized with chloro and amino functional groups. The C-terminal amino acid incorporation, Nα-Fmoc deprotection, acylation reactions and removal of target peptide from the support were optimized. The efficiency of the resin was demonstrated by synthesizing leucyl-alanyl-glycil-valine, alanine-alanine-alanine-alanine, acyl carrier protein (65–74) and retro-acyl carrier protein (74–65) fragments under optimal conditions and is compared with Merrifield resin. The purity of the peptides was checked by HPLC and identities were established by amino acid analysis and mass spectroscopic techniques.

Keywords: GDMA-PMMA resin; Suspension polymerization; Solid phase synthesis

1. Introduction

Polymer-supported reactions have received much consideration since the innovative work of Merrifield on the solid phase synthesis of polypeptides [1]. The solid phase technique is widely used in biopolymer synthesis, small molecule organic synthesis and in combinatorial chemistry [2–4]. The use of polymers is indeed, extremely not only for the synthesis of very complex molecules or even one step processes where the polymer acts as a simple chemical reagent or catalyst [5,6]. The DVB-cross-linked polystyrene support shows rigidity and high mechanical strength. It swells well in non-polar solvents than the polar solvents because of its high hydrophobic macromolecular network [7]. The resins presently used for solid phase organic synthesis are constructed predominantly from cross-linked polystyrene. The synthesis of hydrophilic polymeric compounds on the PS-DVB resin is not so much effective because of its aggregation in non-polar solvents. The compatibility between the growing polymeric chain and the support can be increased by introducing polar polyacrylamide type supports [2,8]. These resins showed effective swelling in polar solvents but their swelling in non-polar solvents is rather poor. The mechanical stability of these resins is very much less compared to polystyrene-based resins. A number of resins were introduced during the last two decades include derivatized PEG-grafted polystyrene-divinylbenzene, monomethyloxy PEG-grafted polystyrene-divinylbenzene (PEG-PS), PEGA, PEO-PS, CLEAR, POEPOP, POEPS, POEPS-3, SPOCC, CLEPSER, HYDRA, PS-TTEGDA, PS-HDODA and PS-BDODMA [9–21]. In the process of selecting a proper support, it is important to consider the optimal performance during solid phase synthesis. For most purposes, the mechanically stable beaded gel resins are preferred. These resins are homogeneous but as a result of functionalization the physical and

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chemical properties can be varied. Solid support with optimal properties has been obtained by radical polymerization of glycerol dimethacrylate (GDMA) with methyl methacrylate.

One of the main challenges in peptide synthesis is to establish synthetic routes to homogeneous products of defined structure. Solid phase peptide synthesis is a synthetic protocol in which a heterogeneous reaction takes place between the solvated resin and the soluble-activated amino acid derivatives in suitable solvents. The polymer support could not be considered as a rigid and inert material in liquid reaction mixture. The chemical and topographical behavior of the polymer support determines its physicochemical properties [22]. The chemical nature of the monomer, mole percentage of cross-linker, type of diluents, geometry of the reaction vessel and organic phase to aqueous phase ratio are the various parameters that determines the yield and topography of the polymer support [23,24]. The support interacts with reaction medium and has significant role in polymer-supported synthesis of polypeptides. If the support and the substrates are compatible, favorable interaction between the polymer and the substrate molecules occur which improves the rate of the reaction. The PS-DVB resin though highly stable, its hydrophobic character can result in unfavorable-coupling reactions. A new GDMA-cross-linked polymethyl methacrylate polymer was introduced as an efficient support for solid phase peptide synthesis. Influence of GDMA cross-linker in the polymethyl methacrylate support for polypeptide synthesis was studied by synthesizing some model peptides. The hydrophilic–hydrophobic balance of glycerol dimethacrylate cross-linked polymethyl methacrylate polymer support (GDMA-PMMA) resin influences its physicochemical properties, and the yield and purity of the peptide reveals the advantages of the resin over conventional resins.

2. Experimental

2.1. Materials

The following reagents were used in the work without additional purification. GDMA, methyl methacrylate, trifluoroacetic acid (TFA), poly(vinyl alcohol) (MW ~ 75,000), benzoyl peroxide and piperidine (Aldrich Chemical Co., USA), 2-(1*H*-benzotriazol-1-yl) 1,1,3,3-tetramethyl uroniumhexafluoro phosphate (HBTU), 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT), *N*-methyl imidazole and Fmoc-amino acids (Novabiochem Ltd, UK). All solvents were purified according to literature procedure before use. GDMA and methyl methacrylate were destabilized prior to use.

IR spectra were recorded on a Shimadzu IR 470 spectrophotometer in KBr pellets. The ^{13}C CP MAS solid state NMR spectra were measured using Bruker 300 MSL CP-MAS instrument operating at 75.47 MHz. HPLC was performed on a Pharmacia Akta purifier instrument (C-18 reversed phase prep. HPLC column) 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 and 100% B in 50 min. The amino acid analysis was carried out on an LKB Alpha plus amino acid analyzer. Mass spectra of the peptides were performed using Kratos MALDI TOF MS instrument.

2.2. Synthesis of GDMA-cross-linked polystyrene (GDMA-PMMA) support

2.2.1. Bulk polymerization

A mixture of methyl methacrylate, GDMA, benzoyl peroxide (0.5 g) and toluene were taken in a 100 ml beaker and agitated using a stirring bar. The reaction mixture was kept in a thermostated oil bath maintained at 80 °C for 30 min. The white, hard bulky copolymer obtained was washed with acetone, chloroform and methanol. The polymer was granulated and sieved. The 100–200 μm fractions were collected and dried under vacuum.

2.2.2. Suspension polymerization

The polymerization was carried out in a conventional suspension polymerization reactor. A 1% solution of poly(vinyl alcohol) was prepared by dissolving PVA (1.1 g) in doubly distilled water (110 ml) at 80 °C. A mixture of methyl methacrylate, GDMA and benzoyl peroxide (0.5 g) dissolved in porogen (10 ml) were added to PVA solution by stirring the aqueous solution at 1000 rpm. A slow stream of nitrogen was bubbled into the reaction mixture. The temperature of the reaction mixture was maintained at 80 °C using a thermostated oil bath and the reaction is allowed to continue for 4 h. The solvent-embedded copolymer beads were washed free of stabilizer and the unreacted monomers by treating with hot distilled water, acetone, chloroform and methanol. The polymer beads were dried under vacuum at 40 °C for 10 h. IR (KBr): 3443 cm^{-1} (OH); 1732.6 cm^{-1} (ester). ^{13}C CP MAS NMR: 19.789 ppm (CH_3 in polymer backbone), 47.527 ppm (backbone $-CH_2-$), 54.661 ppm ($-CH_2-$ of cross-linker), 67.469 ppm (OH bearing C), 180.729 ppm (carbonyl carbon of ester).

The resin (200 mg) was acetylated with measured amount of acetic anhydride–piperidine mixture (1:4, 3 ml) for 6 h. Add 10 ml distilled water and refluxed for 3 h and the mixture was cooled and filtered. Acetic acid formed was back titrated with standard (0.1N) NaOH. The hydroxyl capacity of the resin can be calculated from the titre values.

2.3. Swelling studies

The solvent imbibitions of various resins were 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 from the volume resulting when a definite weight of dry resin was added to a known volume of solvent in a small measuring cylinder.

2.4. Stability studies

The stability studies of the resin were carried out in different reagents such as aqueous hydroxylamine (10 ml), aqueous ammonium hydroxide (10 ml), 20% piperidine in DMF (10 ml), 30% TFA in DCM (10 ml) and 100% TFA (10 ml). The resin samples, 100 mg of each were separately stirred with the above reagents. After 48 h, the resin samples were filtered, washed with ethanol, water, acetone, DCM, dioxane and ether (each 3 × 10 ml), dried and IR (KBr) spectra of these resins were compared with the original.

2.5. Chloro-2%GDMA-PMMA resin

The 2%GDMA-PMMA resin (0.15 mmol/g, 2 g) was swollen in DCM (50 ml). After 1 h, the excess DCM was filtered off. The $SOCl_2$ (6 mmol, ml) was added dropwise to the swollen resin with occasional swirling at 50 °C. After 3 h, the resin was filtered, washed with DCM, DMF, dioxane, ethanol and methanol (each 3 × 20 ml) and dried in vacuum. The amount of chlorine substituted was determined by Volhardt's method [25] with chlorine capacity 0.14 mmol/g.

2.6. Amino-2%GDMA-PMMA resin

The chloro-2%GDMA-PMMA resin (0.14 mmol Cl/g, 2 g) was swollen in DMF (50 ml). After 2 h, the resin was filtered and stirred with potassium phthalimide (518.65 mg, 2.8 mmol) in DMF (20 ml) at 120 °C for 12 h. The resin was filtered, washed with DMF (3 × 20 ml), dioxane (3 × 20 ml), ethanol (3 × 20 ml) and methanol (3 × 20 ml). The dried resin was suspended in ethanol (20 ml) and refluxed with hydrazine hydrate (87.2 μl, 2.8 mmol). After 8 h, the resin was filtered, washed with hot ethanol (3 × 20 ml) and methanol (3 × 20 ml) and dried in vacuum. The amino capacity of the resin is 0.12 mmol/g as estimated by picric acid titration method [26].

2.7. Esterification of Fmoc-amino acid to polymer support using MSNT

The cross-linked resin (1 equiv.) was swelled in dry DCM. Dry Fmoc-amino acid (2 equiv.) in a septum-stoppered flask was dissolved in dry DCM using appropriate volume of dry THF. This solution was transferred to a stoppered flask containing MSNT (2 equiv.). The mixture was immediately added to the swollen resin. After 30 min, the reactants were washed off with DCM, DMF, MeOH and ether (each 5 × 25 ml). The Fmoc-protection was removed by 20% piperidine/DMF and coupling yield was determined from the optical density (OD) of dibenzofulvene-piperidine adduct at 290 nm.

2.8. Time-dependent Fmoc deprotection

The Fmoc-amino acid anchored resin (250 mg) was treated with 20% piperidine/DMF (10 ml). About 10 mg of the resin was withdrawn from the reaction mixture at 2 min interval upto 30 min and the resins were washed with DMF (5 × 10 ml), MeOH (5 × 10 ml), ether (5 × 10 ml) and dried. Accurately weighed resin was treated with 0.1 M picric acid and the extent of Fmoc deprotection was measured from the OD of the picrate adsorbed on the resin at 358 nm. This was further confirmed by suspending 5 mg of the partially Fmoc cleaved resin in 3 ml 20% piperidine in DMF for 30 min. The percentage cleavage was estimated by measuring the OD of the dibenzofulvene-piperidine adduct at 290 nm.

2.9. Time-dependent incorporation of amino acids at different temperatures

The Fmoc protection of C-terminal amino acid attached resin was removed by suspending the resin in 20% piperidine in DMF (10 ml) for 10 min. The resin was washed with DMF (5 × 10 ml). Fmoc-amino acid (3 equiv. relative to the amino capacity), HOBt (3 equiv.) and HBTU (3 equiv.) in DMF (2 ml) were shaken with the resin. About 5 mg of the resin were withdrawn from the reaction mixture at every 5 min upto 1 h. The resin was washed with DMF (6 × 30 ml), MeOH (5 × 25 ml) and ether (5 × 25 ml) and dried. The Fmoc content in the resin was determined by measuring the OD of the dibenzofulvene-piperidine adduct. The same protocol was used for the optimization of coupling rate of amino acids at 40 and 50 °C. The reaction was carried out by wrapping the reaction vessel in thermolyne heating tape and was regulated with a rheostat.

2.10. Detachment of peptide from the GDMA-PMMA resin

The peptidyl resin (Leu-Ala-Gly-Val-resin) was treated with TFA (2.85 ml) and water (150 μl) for 1–18 h at 30, 40 and 50 °C. The yield of the peptide was calculated by comparing the weight of the peptidyl resin and the amount of peptide obtained.

2.11. General procedure for peptide synthesis

The peptides were synthesized using Fmoc-amino acids. All the Fmoc-amino acids were coupled to the C-terminal

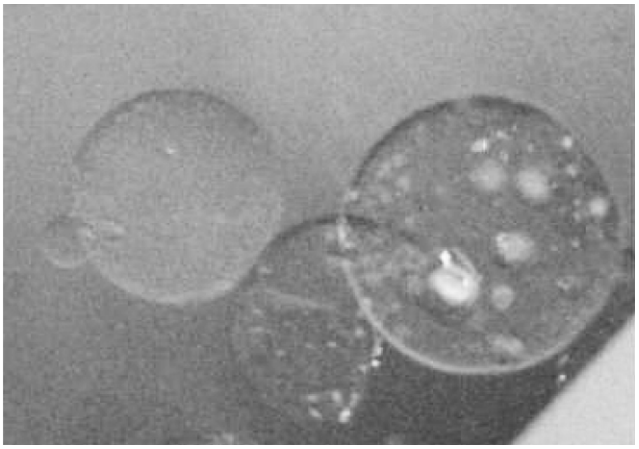


Fig. 1. Optical micrograph of GDMA-PMMA resin.

amino acid attached resin (1 equiv.) by using HBTU and HOBt. In a typical coupling step HBTU (2.5 equiv.) and HOBt (2.5 equiv.) were added to the Fmoc-amino acid dissolved in DMF (1 ml). The mixture was stirred and added to the resin swollen in DMF and the reaction was continued for 30 min. The extent of coupling was monitored by the Kaiser test. Fmoc protection was removed by using 20% piperidine in DMF. After each coupling and Fmoc deprotection steps, the resin was washed with DMF (5 × 50 ml). When the desired sequence of amino acids were attached to the resin, washed

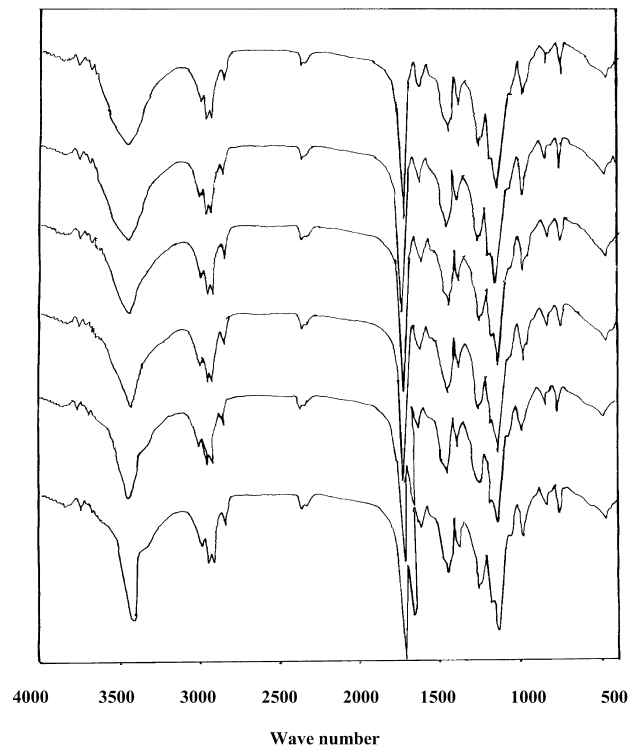


Fig. 2. IR (KBr) spectra of GDMA-PMMA: (a) original; after 48 h treatment with (b) aqueous hydroxylamine; (c) aqueous ammonium hydroxide; (d) 20% piperidine in DMF; (e) 30% TFA in DCM and (f) 100% TFA.

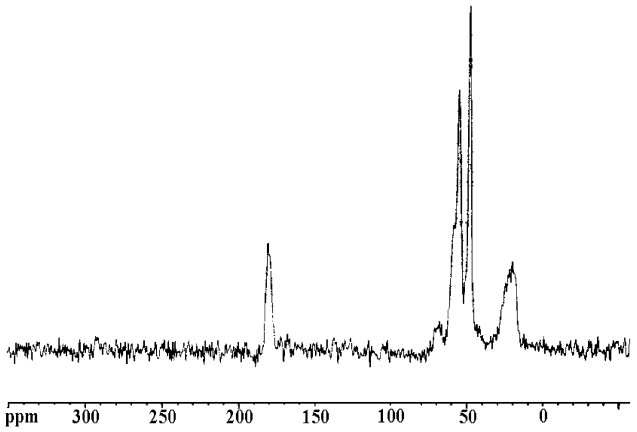


Fig. 3. ^{13}C CP MAS NMR spectrum of GDMA-PMMA resin.

with DMF (5 × 50 ml), MeOH (5 × 50 ml), and ether (5 × 50 ml) and dried under vacuum.

The peptidyl resins were treated separately with TFA (2.85 ml) and water (150 μl) for 6 h at 30 °C. The yield of peptide was calculated by comparing the weight of the peptidyl resin and the amount of peptide obtained.

2.11.1. Leu-Gly-Ala-Val

The yield of crude peptide from the GDMA-PMMA resin is 13.4 mg (97%, based on the C-terminal Val incorporated to the resin). Amino acid analysis: Val, 1.03 (1); Gly, 1.0 (1); Ala, 1.1 (1); Leu, 0.98 (1). MALDI TOF MS: *m/z* 360.1 [$(M + H)^+$, 100%] $C_{16}H_{30}N_4O_5$ requires M^+ 358.43. The yield of crude peptide from the PS-DVB resin is 10.9 mg (85%).

2.11.2. Ala-Ala-Ala-Ala

The yield of crude peptide from the GDMA-PMMA resin is 11.9 mg (94 %, based on the C-terminal Ala incorporated to the resin). Amino acid analysis: Ala, 4.2 (4). MALDI TOF MS: *m/z* 303.16 [$(M + H)^+$, 100%] $C_{12}H_{22}N_4O_5$ requires M^+ 302.336. The yield of crude peptide from the PS-DVB resin is 9.4 mg (83%).

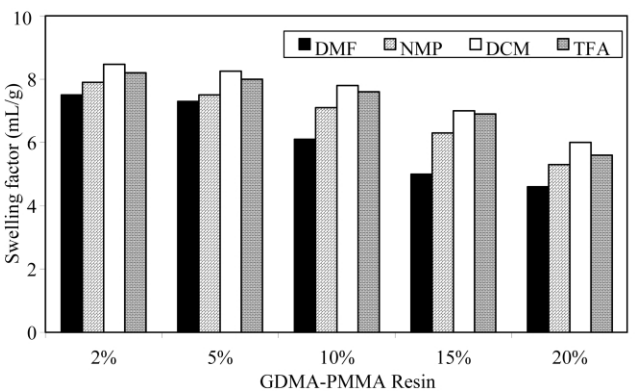


Fig. 4. Swelling comparison of GDMA-PMMA resin with various cross-linking densities in different solvents.

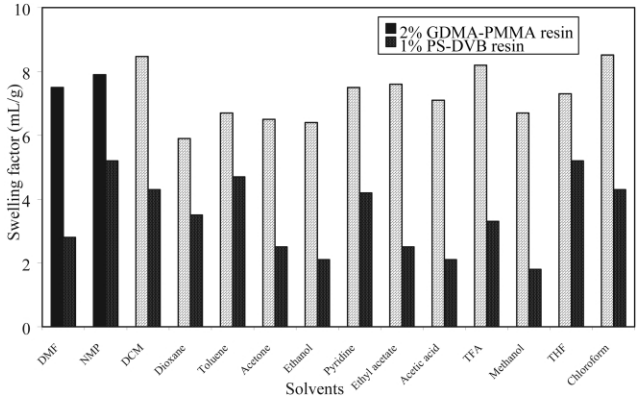


Fig. 5. Swelling comparison of 1% PS-DVB with 2% GDMA-PMMA resin.

2.11.3. Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly (ACP peptide 65–74 fragment)

The yield of crude peptide from the GDMA-PMMA resin is 38.7 mg (95%, based on the C-terminal Gly incorporated to the resin). Amino acid analysis: Gly, 1.1 (1); Ile, 2.01 (2); Tyr, 0.65 (1); Asp, 2.13 (2); Ala, 2.09 (2); Glu, 0.96 (1); Val, 1.0 (1). The low value of Tyr is due to the partial degradation. Gln and Asn were hydrolyzed to Glu and Asp. MALDI TOF MS: *m/z* 1046.84 $[(M + H)^+$, 100%] $C_{47}H_{74}N_{12}O_{16}$ requires M^+ 1045.12. The yield of crude peptide from the PS-DVB resin is 27 mg (65%).

2.11.4. Gly-Asn-Ile-Tyr-Asp-Ile-Ala-Ala-Gln-Val (retro-ACP peptide 74–65 fragment)

The yield of crude peptide from the GDMA-PMMA resin is 35.2 mg (92%, based on the C-terminal Val incorporated to the resin). Amino acid analysis: Gly, 1.01 (1); Ile, 1.98 (2); Tyr, 0.69 (1); Asp, 2.03 (2); Ala, 1.99 (2); Glu, 0.95 (1); Val, 1.1 (1). The low value of Tyr is due to the partial degradation. Gln and Asn were hydrolyzed to Glu and Asp. MALDI TOF MS: *m/z* 1046.53 $[(M + H)^+$, 100%] $C_{47}H_{74}N_{12}O_{16}$ requires M^+ 1045.12. The yield of crude peptide from the PS-DVB resin is 23 mg (62%).

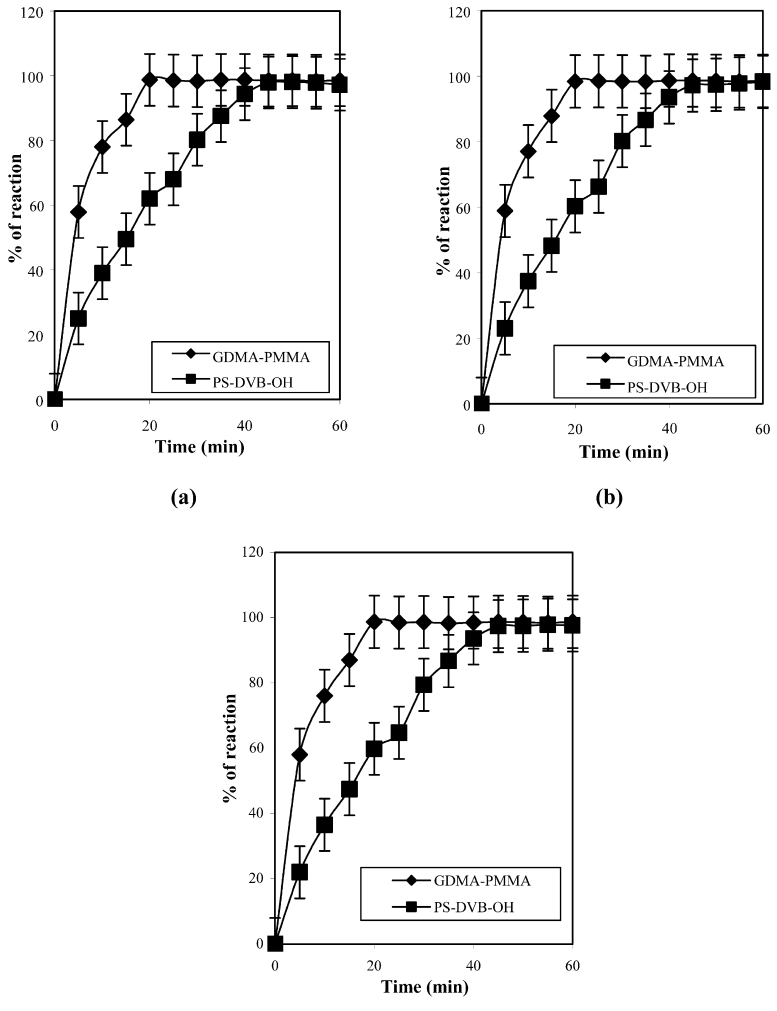
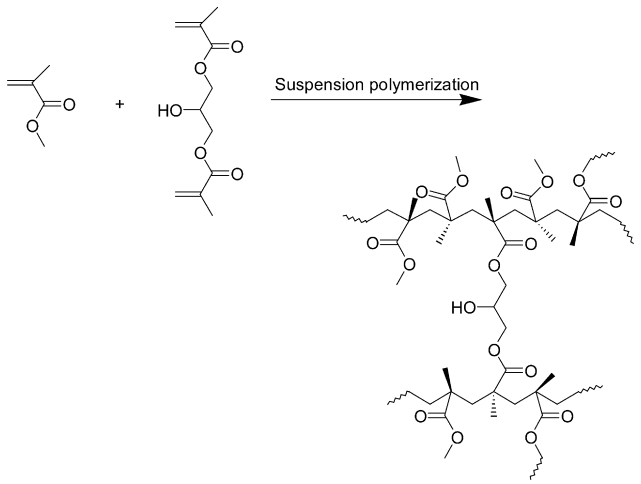


Fig. 6. Time-dependent incorporation of C-terminal amino acids: (a) Fmoc-Ala; (b) Fmoc-Gly; (c) Fmoc-Val.



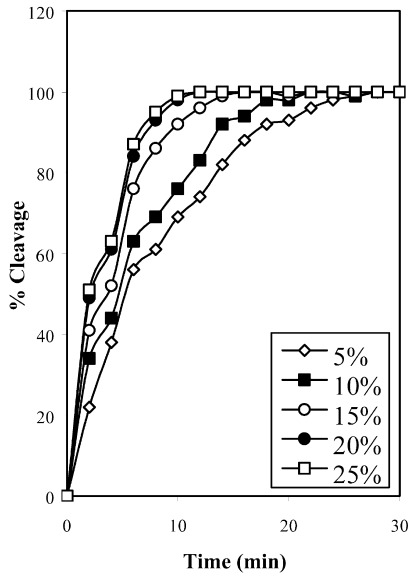
Scheme 1. Preparation of GDMA-cross-linked polymethyl methacrylate resin.

3. Results and discussion

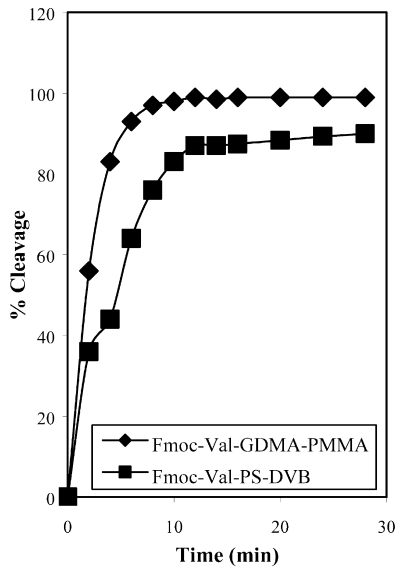
3.1. Synthesis of GDMA-cross-linked polystyrene (GDMA-PMMA) support

The important parameters, which 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, which provides the desired mechanical integrity and polarity to the resin. GDMA-PMMA support showed 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 biopolymers such as peptide.

In the initial phase of this work, bulk polymerization of GDMA and methyl methacrylate were carried out using toluene as the porogenic solvent. The polymer obtained after polymerization was subsequently crushed and sieved to particles of below 200 mesh size. The grinding process is unsatisfactory for several reasons. It inevitably produces irregular particles as well as a considerable quantity of fine particles, which have to be removed by sedimentation. Typically less than 50% of the ground polymer is recovered as useable particles. 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 product which lends itself to further conversions [27,28]. The polymer was synthesized with various cross-linking densities (2, 5, 10, 15 and 20%) by the free radical aqueous suspension polymerization using different porogens such as toluene, carbon tetrachloride, ethyl acetate and cyclohexane (Scheme 1). The insoluble polymer support was obtained as spherical uniform beads. When toluene is used as the



(a)



(b)

Fig. 7. (a) Time-dependent Fmoc-removal from C-terminal Fmoc-Val attached GDMA-PMMA resin with various concentrations of piperidine in DMF. (b) Time-dependent Fmoc-removal of C-terminal Fmoc-Val from GDMA-PMMA and PS-DVB supports using 20% piperidine in DMF.

diluent, the main fractions of the polymer beads are in 100–200 mesh size (Table 1). Reproducible results were obtained by adjusting the amount of stabilizer PVA, geometry of the vessel and stirrer and the stirring rate. The yield and size of the beaded polymer with 2 mol% GDMA using different diluents are given in Table 2. The optical micrograph of the polymer showed that they are uniform spherical beads and the surface is even and smooth (Fig. 1).

IR and ^{13}C CP MAS NMR spectroscopic techniques are used for the characterization of GDMA-PMMA resin. The resin was powdered and palletized with KBr. IR spectrum

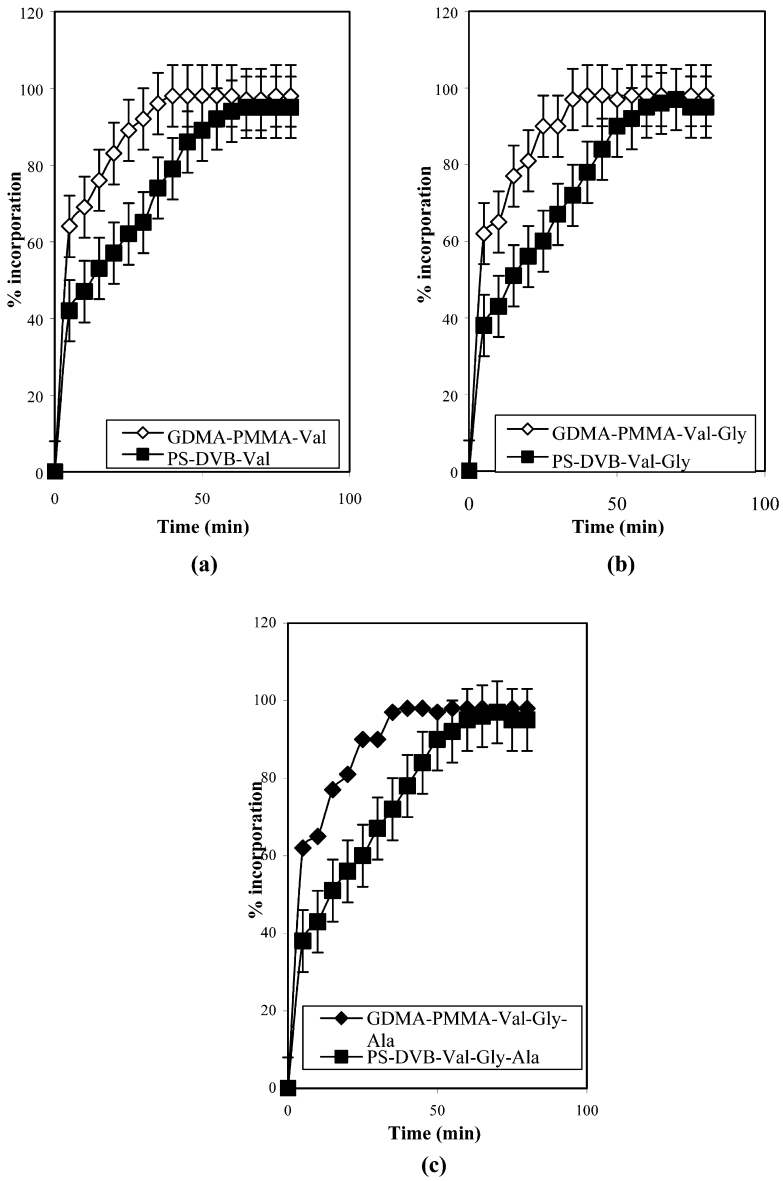


Fig. 8. Time-dependent acylation at room temperature.

gave a sharp and intense peak at 1732.60 cm^{-1} corresponding to ester carbonyl group of the cross-linker in addition to the peaks of polymethyl methacrylate (Fig. 2(a)). ^{13}C CP MAS NMR spectrum indicates a small peak at 19.789 ppm corresponding to the CH_3 in polymer backbone. The peaks at 47.527 and 54.661 ppm corresponds to the $-CH_2-$ backbone and cross-linker. The peak at 67.469 represents the OH bearing carbon in the cross-linker. The intense peak at 180.729 ppm indicates the carbonyl carbon of ester group (Fig. 3).

3.2. Swelling and stability studies of polymer support

The chemical stability of the resin in various solvents and

Table 1
Preparation of GDMA-PMMA resin by suspension polymerization (toluene is used as the diluent)

GDMA in feed (mol%)	Amount of methyl methacrylate (g)	Amount of GDMA (g)	Yield of polymer (g)	OH capacity (mmol/g)
2	9.81	0.45	8.73	0.14
5	9.51	1.14	9.27	0.38
10	9.01	2.28	10.27	0.77
15	8.51	3.42	11.09	1.1
20	8.00	4.57	11.94	1.45

Table 2
The yield and bead size of polymer obtained using different diluents (2% GDMA in feed)

Diluent	Yield	Bead size (μm)
Toluene	8.74	100–200
Chloroform	8.24	50–100
Ethyl acetate	7.97	50–100
Cyclohexane	8.73	20–50

reagents is one of the factors that determine the efficiency of the support in polypeptide synthesis. Apparently the 2% GDMA-PMMA resin is stable enough to withstand various chemical reactions. The support showed comparable physical and mechanical properties to that of PS-DVB support, permitting identical manipulations such as shaking and filtration when used in SPPS. In order to verify the utility of the resin in peptide synthesis, the stability of the resin is tested in commonly encountered peptide synthetic conditions (Fig. 2). The treatment of the resin with 20% piperidine in DMF (reagent used for Fmoc-removal) for 48 h does not show any change in its IR spectrum indicating that it has enough stability in peptide synthetic conditions using Fmoc-amino acids. The resin do not show any considerable change in IR spectrum even after treatment with aqueous NH_2OH and NH_4OH for 48 h. The IR spectrum of the resin after 48 h treatment with 30% TFA/DCM mixture (reagent used for Boc removal) and 100% TFA shows an additional absorption band at 1680 cm^{-1} corresponding to the carboxyl carbonyl group, indicating that the methyl ester group in the resin cannot withstand the conditions of peptide synthesis using Boc-amino acids. The stability studies showed that the new resin is suitable for peptide synthesis using Fmoc-amino acids.

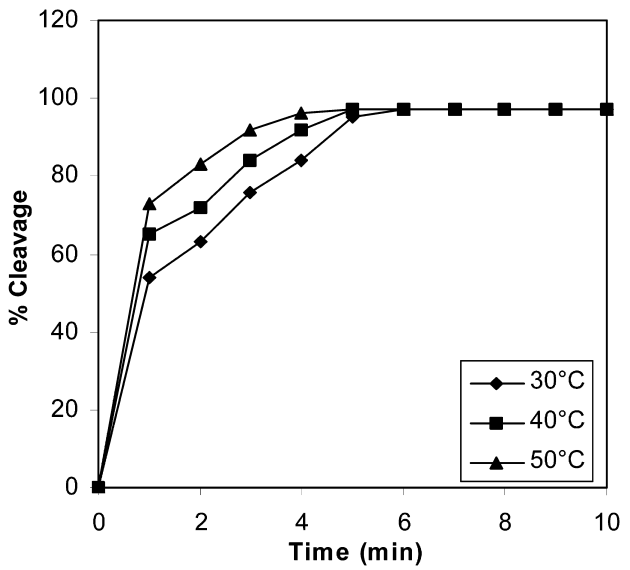


Fig. 9. Time-dependent cleavage of Leu-Ala-Gly-Val at various temperatures.

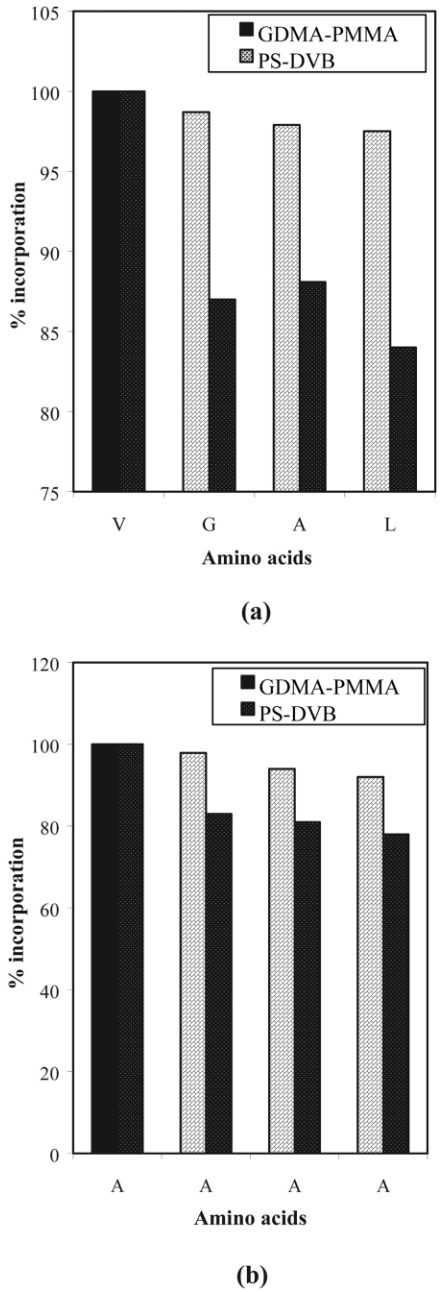


Fig. 10. Percentage incorporation of amino acids: (a) Leu-Ala-Gly-Val; (b) Ala-Ala-Ala-Ala.

In solid phase synthesis, the accessibility of the resin-bound substrate to reagent and solvents is very important. For maximum accessibility of the reactive functional group in the resin, the polymer matrix should swell extensively in the solvating medium [29]. The extent of swelling of the resin was a measure of its solvation by a given solvent [30,31]. The swelling efficiency of the resin decreases with increase in cross-linking density.

The solvent imbibitions of the various cross-linked GDMA-PMMA resins was determined by a centrifuge method and/or by placing the resin in graduated cylinders with excess solvent and noting the initial and final volume

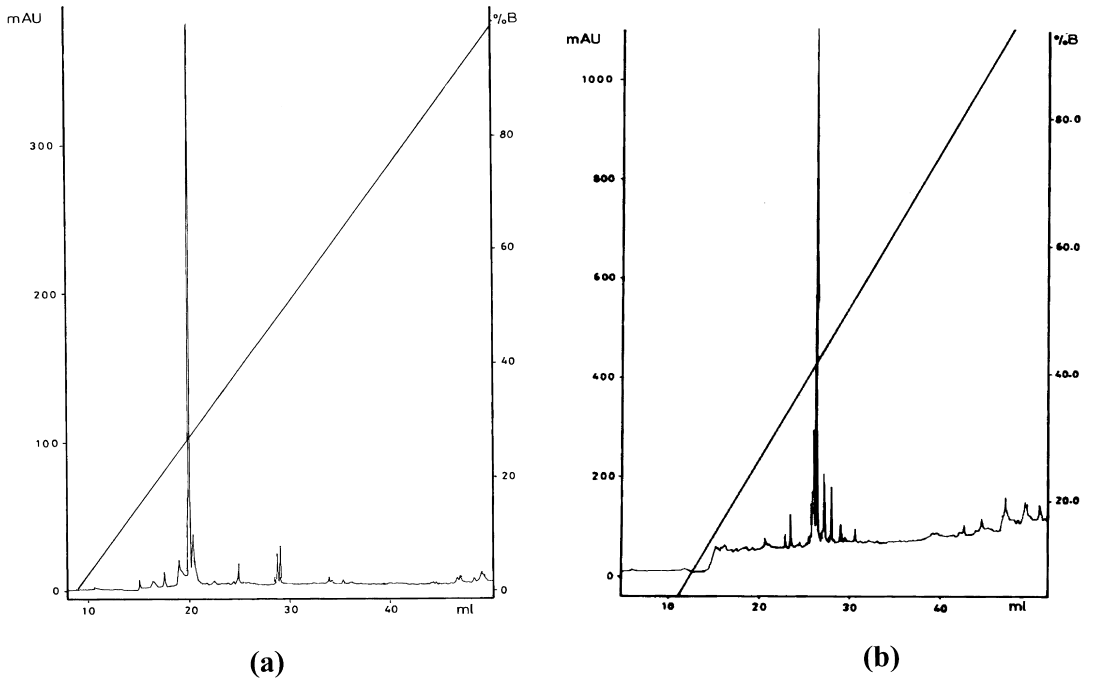


Fig. 11. HPLC analysis of peptide Leu-Ala-Gly-Val: (a) GDMA-PMMA; (b) PS-DVB.

of the bead. Though the 1% GDMA-PMMA resin showed very high swelling property in various solvents, it was not used for the synthesis of peptides because of its pulverization when the number of amino acids exceeds 10. This problem also results in difficulties when the reagents and solvents used for the synthesis were removed by filtration. The GDMA-PMMA resins with more than 5% cross-linking density are not used as supports for solid phase peptide synthesis. These resins are rigid and their swelling characteristics decrease as the increase in cross-linking density. The resins with more than 5% cross-linker have a higher hydroxyl capacity and causes steric hindrance during peptide synthesis.

The 2–5% GDMA-PMMA resins are found to be good

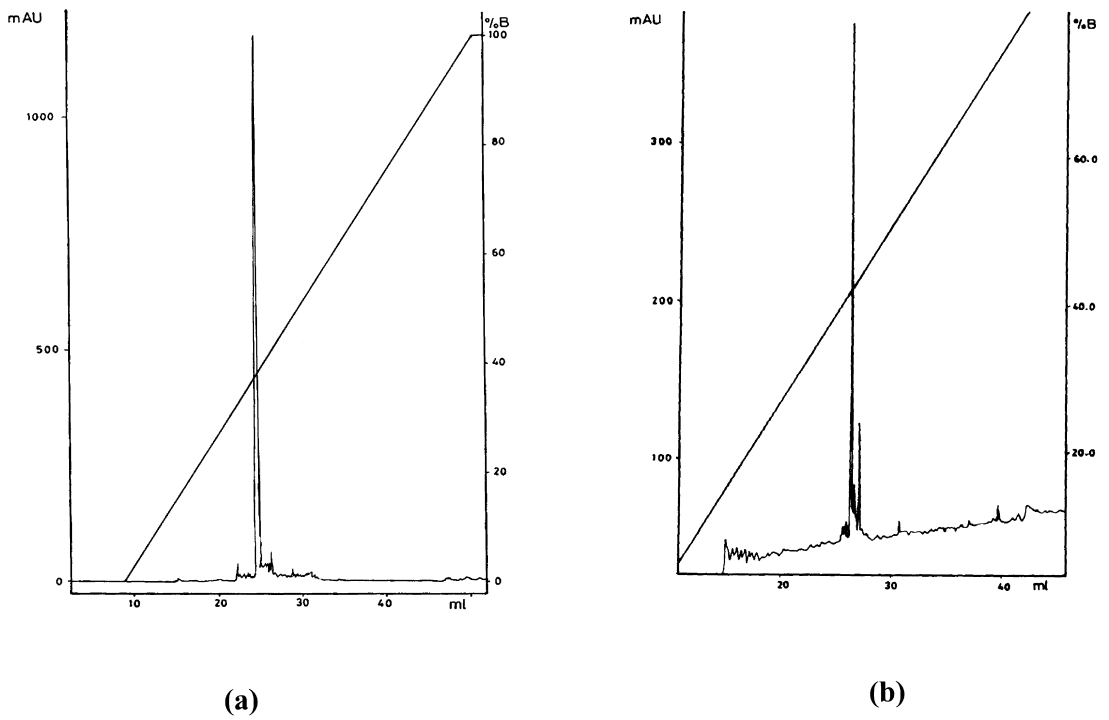


Fig. 12. HPLC analysis of peptide Ala-Ala-Ala-Ala: (a) GDMA-PMMA; (b) PS-DVB.

support for the synthesis. Since 2% cross-linked support showed better swelling characteristics compared to 3–5% cross-linked supports in different solvents, it was selected for the synthesis of peptides. The 2% GDMA-PMMA resin showed extensive swelling in various solvents compared to 1% PS-DVB resin and GDMA-PMMA resins with higher cross-linking densities. To investigate the effect of the hydrophilic cross-linking agent, the swelling characteristics of the resin in solvents of varying polarity were measured. The results of swelling studies are given in Figs. 4 and 5. The results showed that the GDMA-PMMA resin has superior swelling characteristics in various polar and non-polar solvents. This can facilitate the diffusion of soluble reactants and reagents into the polymer matrix and increases the rate of the reaction.

3.3. Time-dependent incorporation of C-terminal amino acid

The time-dependent percentage incorporation of C-terminal amino acid of the respective peptides (Fmoc-Val, Fmoc-Ala and Fmoc-Gly) on GDMA-PMMA resin was compared with hydroxymethyl PS-DVB resin (Fig. 6). The MSNT-coupling method was used for the attachment of C-terminal Fmoc-amino acid to the resins. The resins having approximately same hydroxyl capacity were treated with 2 equiv. of MSNT, Fmoc-amino acid and 1.5 equiv. of *N*-methylimidazole. The GDMA-PMMA resin required 20 min for quantitative reaction, whereas hydroxymethyl PS-DVB resin required 45 min. This discrepancy appears to be due to the flexible, hydrophilic GDMA cross-linker and polymethyl methacrylate backbone of the resin compared to the hydrophobic DVB cross-linker and polystyrene backbone in PS-DVB resin that can allow free interaction between the reactive centers on the support and the respective amino acids and reagents in DMF.

3.4. Time-dependent Fmoc deprotection

The time-dependent study of Fmoc-removal using 5, 10, 15, 20 and 25% piperidine in DMF from the C-terminal amino acid of GDMA-PMMA showed that the percentage cleavage in unit time increases with base concentration (Fig. 7(a)). The results showed that 20 and 25% base concentrations are suitable for the Fmoc-removal. But 20% base concentration is preferred due to avoid the side reactions during higher base concentration. The rate of cleavage of Fmoc group with 20% piperidine in DMF was determined by measuring the OD of the dibenzofulvene-piperidine adduct. The GDMA-PMMA resin required 15 min, whereas PS-DVB resin required 30 min for quantitative removal. The results showed that the reaction rate in GDMA-PMMA resin is twice that in PS-DVB resin (Fig. 7(b)). The higher solvation and swelling characteristics of GDMA-PMMA resin in the reaction medium may enhance the free interaction of the protected amino acids and cleavage reagent improving the reaction rate.

3.5. Comparative synthesis of peptides

Merrifield's model peptide was synthesized on GDMA-PMMA and PS-DVB resins. A comparative time-dependent acylation at room temperature (30 °C) was carried out on these resins. The extent of reaction in the first 10 min was higher for GDMA-PMMA and showed about 20% increase in coupling efficiency compared to PS-DVB resin (Fig. 8). A comparative time-dependent cleavage of peptide at room temperature showed 97% cleavage in 6 h from the GDMA-PMMA resin, whereas PS-DVB resin required 20 h for 85% cleavage (Fig. 9(a)). The duration of cleavage of the peptide decreases as the temperature increases (Fig. 9(b)). The HPLC profiles of the peptide obtained at higher temperatures showed some additional peaks.

The efficacy of the GDMA-PMMA resin over PS-DVB resin was confirmed by the synthesis of peptides alanyl-alanyl-alanyl-alanine 65–74 fragment of ACP and retro-ACP (74–65) fragments using Fmoc-amino acids. The

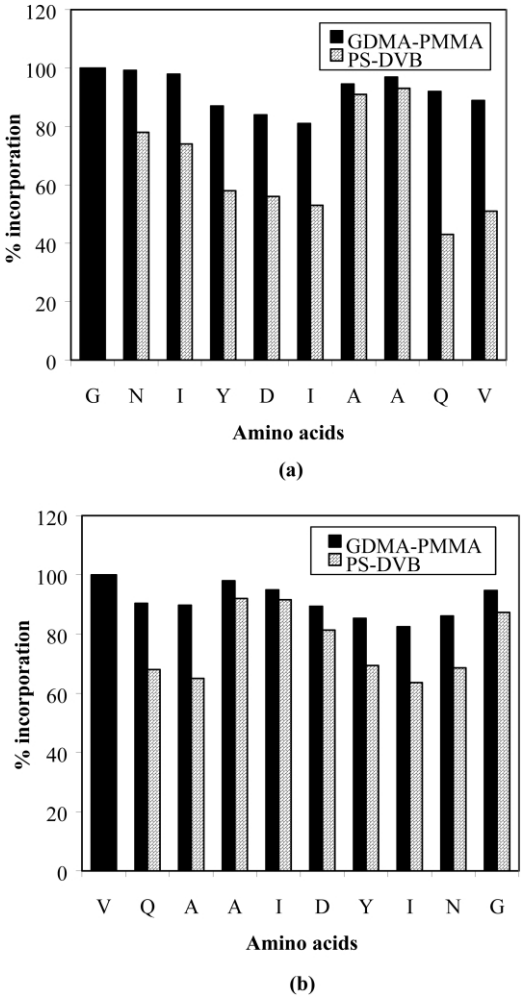


Fig. 13. Percentage incorporation of amino acids: (a) ACP; (b) retro-ACP.

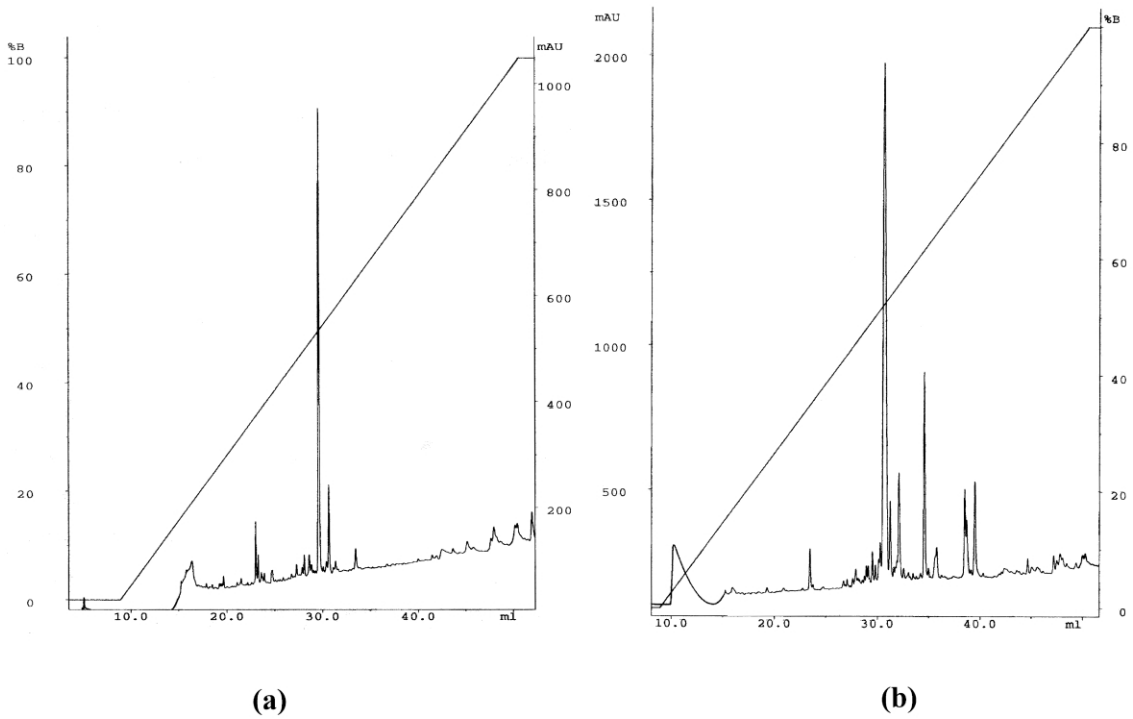


Fig. 14. HPLC profile of peptide ACP: (a) GDMA-PMMA; (b) PS-DVB.

percentage incorporation of amino acids of the peptide Ala-Ala-Ala-Ala in GDMA-PMMA and PS-DVB resins was monitored under identical conditions (Fig. 10(b)). The amino capacity of the final peptidyl resin (GDMA-PMMA) showed that 99.4% of the theoretically estimated peptide chains were retained in the resin. The yield of the crude peptide from 200 mg of the starting resin was 94% of the theoretically calculated value. The HPLC analyzing data of the peptide synthesized on GDMA-PMMA showed only a sharp single peak with a percentage of pure peptide was 93.5. The chromatogram of the peptide from PS-DVB resin showed some additional peaks along with the major peak (percentage of pure peptide calculated from the peak area was 68 (Figs. 11 and 12). The percentage incorporation

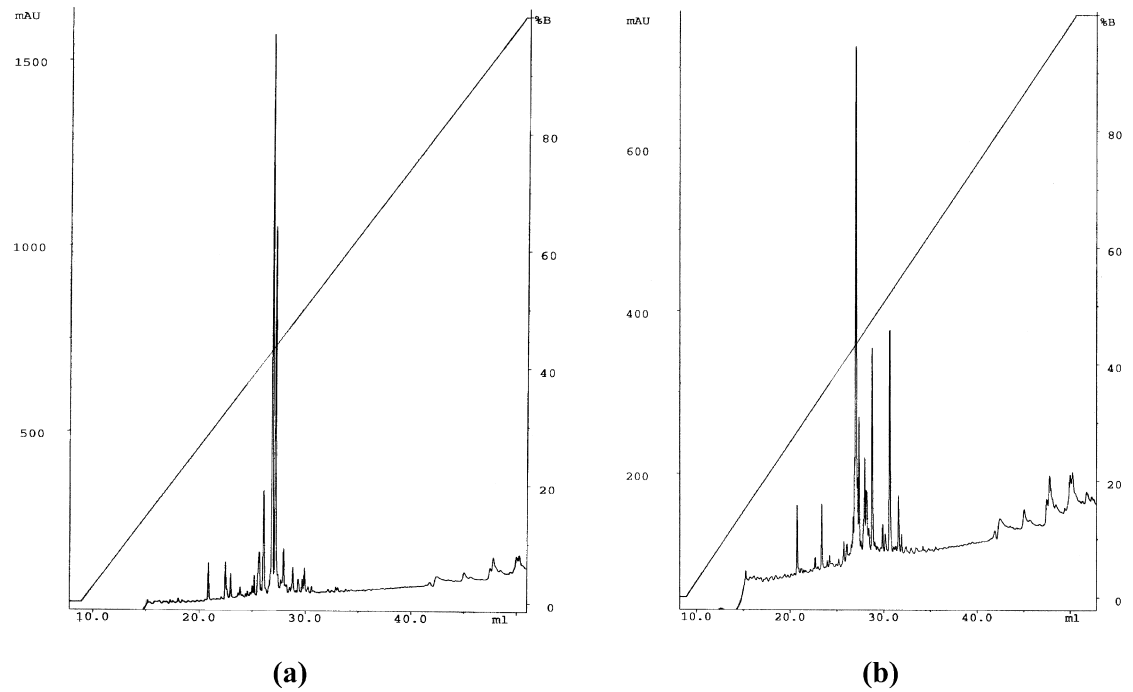


Fig. 15. HPLC profile of peptide retro-ACP: (a) GDMA-PMMA; (b) PS-DVB.

studies of amino acids of ACP (Fig. 13(a)) and retro-ACP (Fig. 13(b)) showed that the reaction proceed quantitatively in GDMA-PMMA resin. The yield of crude ACP and retro-ACP fragments from the 200 mg of GDMA-PMMA and PS-DVB resins are 95 and 92, and 65 and 63%, respectively. From the high performance liquid chromatogram, the peak area corresponding to ACP fraction showed that the GDMA-PMMA yielded 73% of pure peptide and PS-DVB resin yielded 38% of pure peptide (Fig. 14). The pure retro-ACP from GDMA-PMMA and PS-DVB are 71 and 35%, respectively (Fig. 15).

4. Conclusions

A new, flexible polymeric support was synthesized by the co-polymerization of GDMA and methyl methacrylate. The support is cost-effective since they are prepared by an easy single step polymerization using readily available low cost monomers. The hydroxyl groups of the polymer support are amenable to a wide range of reactions without affecting the polymer matrix. The structure of the polymer provides easy diffusion of reagents and solvents through the resin matrix. The anchoring of functional groups and their reactivity were highly enhanced due to the greater chain mobility of GDMA cross-link and polymethyl methacrylate matrix compared to rigid hydrophobic PS-DVB support. The new flexible GDMA-PMMA resin can be used for the synthesis of hydrophobic peptides in very high yield and purity.

Acknowledgements

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