1,6-Hexanediol Diacrylate-Crosslinked Polystyrene: Preparation, Characterization, and Application in Peptide Synthesis

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ABSTRACT: A copolymer of 1,6-hexanediol diacrylate (HDODA) and styrene was prepared by a suspension polymerization method. The resin was characterized by infrared and carbon-13 cross-polarization magic-angle spin (^{13}C CP-MAS) spectroscopy. The topology of the resin was examined by scanning electron microscopy (SEM). The polymer swells extensively in common solvents used for peptide synthesis. The resin exhibited chemical stability even in neat trifluoroacetic acid. The applicability of the new resin was demonstrated by synthesis of Val-Ala-Val-Ala-Ala-Gly, Gln-Val-Gly-Gln-Val-Glu-Leu-Gly, and Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly. Comparative synthetic studies showed that the new resin is superior to divinylbenzene (DVB)-based resin in the case of the synthesis of hydrophobic peptide sequences. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 87: 1290–1296, 2003

Key words: resins; peptides; polystyrene; polymerization

INTRODUCTION

Solid-phase strategy, which was developed initially as a rapid way to synthesize peptides and oligopeptides,[1,2] has been one of the most powerful approaches to progress of the drug discovery process and the development of compound libraries.[3–5] The key factor in the success of solid-phase synthesis is the right choice of polymeric support. The use of divinylbenzene (DVB)-crosslinked polystyrene (DVB–PS) in many cases is accompanied by difficulty during synthesis. This difficulty is primarily due to the high hydrophobic environment of the support. In addition, the crosslinker (DVB) connecting the PS backbone is short and rigid. Thus, the resin shows affinity towards only a limited range of solvents. The easy diffusion of reagent is favored only when the polymer is in a highly swollen state. However, the swelling tendency of the polymer is counterbalanced by the elastic restraining force exerted by the short and rigid DVB crosslinker.[6] Because the reactions in a solid-phase medium are heterogeneous in nature, the use of different solvents of varying polarity considerably affects the swelling of the polymer, eventually leading to a low yield of the target product.

The developments of poly(ethylene glycol) (PEG)-grafted PS resin[7–10] or PS crosslinked with PEG moiety[11–16] are some of the approaches to circumvent the problems associated with DVB-based resins. Recently, we have shown the utility of 1,4-butanediol dimethacrylate–crosslinked PS (BDDMA–PS) in solid-phase peptide synthesis (SPPS). This resin possesses better solvation and swelling characteristics than DVB–PS.[17] The resin offers a better yield of peptide compared with conventional supports. Here we describe the preparation and characterization of 1,6-hexanediol diacrylate-crosslinked PS (HDODA–PS) and its application in the synthesis of peptides. The utility of the resin is illustrated by the synthesis of three short model peptides. Comparative studies of the resin with DVB–PS show that the resin is an ideal support for the synthesis of even difficult sequences.

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EXPERIMENTAL SECTION

Materials and methods

All side-chain-protected Boc amino acids (L) were procured from Peninsula Company (San Carlos, CA). Simple amino acids (L; Merck, Germany) were Boc protected by a literature procedure.[18] Boc carbazate, hydroxybenzotriazole (HOBt), dicyclohexyl carbodiimide (DCC), and *N*-methyl-2-pyrrolidone (NMP) were obtained from Sigma Chemical Company (San Carlos, CA). HDODA, trifluroacetic acid (TFA), diisopropylethyl amine (DIEA), dimethyl sulphoxide (DMSO), $CsCO_3$, thioanisole, *m*-cresol, triethyl amine (TEA), and styrene were obtained from Aldrich Chemical Company (Milwaukee, WI). All solvents were commercial grade and purified before their use. Infrared (IR) spectra of the polymer samples were taken

with a Shimadzu spectrophotometer (IR 470). Amino acid analyses were performed on a LKB 4151 ALPHA PLUS amino acid analyzer using ninhydrin detection. Optical density (OD) was measured with a Shimadzu ultraviolet–visible (UV–vis) spectrophotometer (UV 160 A) at 358 nm. High-performance liquid chromatography (HPLC) analyses were conducted using a Pharmacia LKB Prep RPC 5/5 system using C18 RP column and binary gradient system (water and acetonitrile containing 0.1% TFA as the solvents). The flow rate was 0.5 mL/min, and detection was at 214 nm (UV). Carbon-13 nuclear magnetic resonance [^{13}C NMR; cross-polarization magic-angle spin (CP-MAS)] spectra of the samples were taken using a dsx 300 (75.47 MHz). The samples were rotated at different frequencies so that the side bands were eliminated.

Synthesis of 2%-crosslinked HDODA-PS

Styrene (10 mL) was destabilized with a 1% NaOH solution (3 × 15 mL). It was then washed with water (3 × 10 mL) and dried using anhydrous calcium chloride. A 1% solution of polyvinyl alcohol (PVA; average mol wt, 75,000) in water (110 mL) was prepared and kept stirred in a polymerization vessel at 80°C, with a constant flow of N_2 through the solution. A mixture of 1,6-hexanediol diacrylate (0.45 mL, 2 mol%), styrene (11.3 mL, 98 mol%), toluene (2.31 mL, 20 vol% of monomer ratio) as diluent, and dibenzoyl peroxide (600 mg) was prepared and added to the PVA solution. The stirring was continued for 8 h. The polymer beads were collected by filtration through a sintered disc (G3) and washed thoroughly with hot water (20 mL × 3, 3 min) to remove PVA, acetone (20 mL × 3, 3 min), and methanol (20 mL × 3, 3 min). The polymer was then Soxblet extracted, using acetone, dichloromethane (DCM), and methanol to remove linear polymers. The yield was 9 g. The polymer beads were sieved, and those of 200–400 mesh sizes were used for peptide synthesis.

Swelling studies

One gram of resin was accurately weighed and placed in a silanized sintered crucible. The crucible size was very small to minimize the error in the studies. Solvent was added to the crucible and, after 5 min, it was kept in a beaker containing the solvent. After 10 h, the solvent was carefully sucked out from the crucibles, and solvent droplets adhering on the crucibles were also removed. The weight increment of the resin due to intake of solvent was noted. The experiment was continued with other solvents and with DVB-PS. All the measurements were carried out at room temperature (28°C). The swelling data were expressed in volume/gram of resin.

Stability of the resin

HDODA–PS resin (2 g) was treated with TFA (10 mL) at 40°C for 48 h. The TFA solution was collected and evaporated. No detectable mass was obtained. The TFA-treated resin was compared with original resin by IR investigation.

Chloromethylation of the polymer support

Anhydrous $ZnCl_2$ (1.5 g) was placed in an Erlenmeyer flask. Concentrated HCl (3 drops) and water (5 drops) were added. The solution was heated until the solid was completely dissolved. Heating was continued until a solid mass of $ZnCl_2$ was left, which melted on further heating. When it became a mobile liquid, the flask was kept in a desiccator and allowed to cool. The solid was dissolved in tetrahydrofuran (THF; 10 mL) and kept sealed.

The dry resin beads (2 g) were kept in DCM (20 mL) in a round bottomed flask. After 0.5 h, chloromethyl methyl ether[19] (CMME, 12 mL) and a solution of $ZnCl_2$ (0.4 mL) in THF was added to the swollen resin. The mixture was kept at 50°C for 5 h. The resin was filtered, washed with THF (20 mL × 3, 3 min), water (20 mL × 3, 3 min), THF–water (1:1 v/v, 20 mL × 3, 3 min), water (20 mL × 3, 3 min), and finally methanol. The resin was dried under vacuum.

Estimation of the extent of chloromethylation

To the chloromethylated resin (200 mg) was added 5 mL of pyridine, and the mixture kept at 110°C for 5 h. The mixture was then quantitatively transferred with acetic acid (1:1 v/v, 20 mL) and diluted with water (25 mL). Concentrated HNO_3 (7 mL) and $AgNO_3$ (0.1 N, 10 mL) were added to this solution and titrated against standard ammonium thiocyanate solution (0.1 N) using ferric alum as indicator. A blank was also performed. Chlorine capacity was 2.0 mmol/g.

Attachment of Boc Gly on HDODA-PS

A saturated solution of cesium carbonate was added to 0.19 g of Boc Gly (1.2 mmol) in ethanol (5 mL) until the pH reached 7.0. The solution was stirred for 2 h, then the ethanol was evaporated under reduced pressure. Water was removed by azeotropic distillation with dry benzene. The white powder of the cesium salt of Boc-Gly was dried under vacuum in the presence of P_2O_5. The salt was dissolved in NMP (2 mL), and to this solution was added 0.3 g of chloromethylated resin (capacity 2.0 mmol/g). The mixture was kept at 50°C for 36 h. The resin was filtered, washed with NMP (5 mL × 3, 3 min), NMP–water (1:1 v/v; 5 mL × 3, 3 min), NMP (5 mL × 3, 3 min), DCM, (5 mL × 3, 3 min), and methanol (5 mL × 3, 3 min). The resin

was dried under vacuum, giving 370 mg of the amino acid resin. The substitution level was 1.9 mmol of Gly/g of resin (by the picric acid method).

Attachment of Boc Gly on DVB-PS

Boc Gly (1.2 mmol, 0.19 g) was dissolved in ethanol (5 mL). The pH of the solution was brought to 7 by the slow addition of a saturated solution of cesium carbonate. The solution was stirred for 2 h, then rotary evaporated to remove ethanol and water. The white powder of cesium salt of Boc Gly was dissolved in NMP (2 mL). DVB-PS (2% crosslinked, 200–400 mesh size, 0.3 g, 2.02 mmol of Cl/g of resin) was added to the solution. The mixture was kept at 50°C for 48 h in an oil bath. The resin was filtered, washed with NMP (5 mL × 3, 3 min), NMP–water (1:1 v/v; 5 mL × 3, 3 min), NMP (5 mL × 3, 3 min), NMP–water (1:1 v/v; 5 mL × 3, 3 min), NMP (5 mL × 3, 3 min), DCM (5 mL × 3, 3 min), and methanol (5 mL × 3, 3 min). Vacuum drying over P_2O_5 afforded 350 mg of amino acid resin. Amino capacity (by the picric acid method) was 1.85 mmol/g.

Amino group estimation: picric acid method

Two milligrams of amino acid resin, after the removal of the Boc group, was treated with 0.1 M picric acid (in DCM). The excess unbound picric acid was removed by washing with DCM (5 mL × 3, 3 min). The resin was treated with 1 mL of 10% TEA (in DCM) and the free picric acid was collected. The resin was washed with 95% ethanol, then was washed again with 10% TEA (1 mL). All the washings were collected and diluted to 15 mL, and the OD at 358 nm was measured. The amino group substitution was calculated from this result on the basis of the standard ε_{max} for picrate–TEA amine complex in ethanol at 358 nm (ε_{max}: 4,500).

Removal of Boc protection: general procedure

The polymer was treated with 30% TFA (in DCM) for 30 min. The TFA solution was filtered and the resin was washed with DCM. This was then treated with 5% DIEA in DCM (5 min) and 5% DIEA in NMP-DCM mixture (1:1 v/v) to yield the free amino acid resin or free peptidyl resin.

Coupling of individual amino acids: general procedure

The pre-formed activated ester method was used for the coupling of amino acids to the free peptidyl resin. The activated ester was prepared using 2.5 mmol each of DCC, HOBt, and amino acid in NMP (4 mL) for 1 mmol of glycine resin. The dicyclohexyl urea (DCU) was filtered off, and the solution was added to the free peptidyl and/or amino resin. The resin was shaken for 45 min in a silanized glass peptide synthesizer. At the end of 45 min, DMSO (0.75 mL) was added to the mixture and, after 15 min, 0.14 mL of DIEA was also added. The total volume of the solution was limited to 5 mL for effective coupling reaction. The solution was filtered, and the resin was washed with a mixture of MeOH and DCM (33;67 v/v) to get rid of DCU and then with a DCM/NMP (1:1 v/v) mixture. The effectiveness of acylation was monitored by the Kaiser test.[20] The protocol adopted for the assembly of one amino acid unit is shown in Table II.

Cleavage of peptide: general procedure

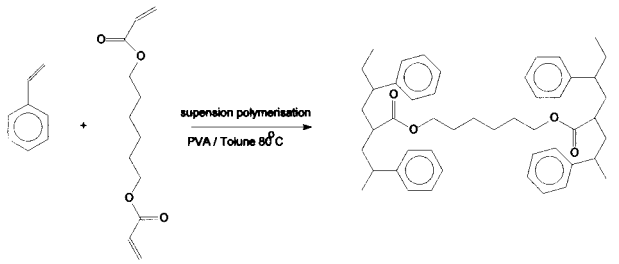
Peptidyl resin (100 mg) was treated with neat TFA (10 mL), thioanisole (0.1 mL), and *m*-cresol (0.1 mL). The mixture was stirred at room temperature for 48 h. The TFA was removed under reduced pressure. The peptide was precipitated by adding cold diethyl ether. The precipitate was washed several times with cold ether and then dried. The peptide was then subjected to HPLC, and the major peak was subjected to amino acid analysis.

Synthesis of the hexapeptide Val-Ala-Val-Ala-Ala-Gly

One-hundred milligrams of the Gly resins (amino capacity: HDODA-PS, 0.19 mmol; DVB-PS, 0.185 mmol) were used for the synthesis. Coupling of subsequent amino acids was performed by the active ester method. The coupling of each amino acid was repeated until the peptide resin showed no response to the Kaiser test (Table II). The weights of peptide–resins were 170 mg for DVB-PS and 190 mg for HDODAA-PS. A 100-mg sample of peptidyl resin was subjected to cleaving conditions. The yields of crude peptides were 39.5 mg (75%) of DVB-PS and 45.5 mg (94%) of HDODA–PS. Results of amino acid analysis of pure peptide are Gly 1(1), Ala 2.9(3), and Val 1.8(2).

Synthesis of the octapeptide Gln-Val-Gly-Gln-Val-Glu-Leu-Gly

A 100-mg sample of the Gly resin was used for the synthesis of the octapeptide according to the protocol shown in Table II. The weights of the peptidyl resins were 210 and 250 mg for DVB-PS and HDODA-PS, respectively. The yields of peptides obtained from 100 mg of each of peptide–resin were 44.7 mg (60%) for DVB–PS and 60 mg (93%) for HDODA–PS. Results of amino acid analysis of pure peptide are Gly 2(2) Val 1.9(2), Glu 2.7(3) and Leu 0.8(1).



Scheme 1 Suspension polymerization of styrene and 1,6-hexanediol diacrylate.

Synthesis of the decapeptide Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly

The synthesis of this peptide was carried out in exactly the same manner as already described. One-hundred milligrams of Gly-resins were used for the synthesis. The weights of peptide–resins were 190 mg for DVB-PS, and 270 mg for HDODA–PS. Eighty-milligrams of peptide–resins were subjected to cleaving conditions. The weights of crude peptides obtained were 25 mg (55%) for DVB-PS and 45 mg (82%) for HDODAA. The result of amino acid analysis of pure peptides are Val 0.62(1), Ala 2.1(2), Glu 0.9(1), Ile 1.95(2), Asp 2.1(2), Tyr 0.99 (1) and Gly 1(1).

RESULTS AND DISCUSSION

Suspension polymerization[21] was used to obtain the polymer in a regular beaded structure (Scheme 1). The copolymerization of styrene and HDODA, with toluene as diluent dispersed as droplets in water was performed in a suspension process. Dibenzoyl peroxide was used as the radical initiator. PVA acts as a protective colloid, giving adequate stabilization to the copolymer droplets. PVA with an average molecular weight of 75,000 Da was effective in the present polymerization. The solubility of high molecular weight PVA was not satisfactory and low molecular weight PVA does not give proper stabilization to the droplets, resulting in coalescence of the copolymer nuclei. Beads of size 200–400 μm were separated and used for the entire study and synthesis. Beads >400 μm have less total surface and beads <200 μm tend to clog the filtering disc of the synthesizer. A scanning electron micrograph (SEM) of the resin shows the regular shape of the bead (Figure 1).

The resin was characterized by IR and ^{13}C NMR analyses. The IR (KBr) spectrum (Figure 2a) of the resin showed peaks at 1490 and 1720 cm^{-1}, which were assigned to the ester-carbonyl. Other prominent peaks were at 3020,700 cm^{-1} (CH of benzene) and 2910, 2850 cm^{-1} (—CH_2 group of styrene and hexanediol diacrylate). The ^{13}C NMR spectrum of the resin (Figure 3) showed a large peak at 130.319 ppm, which was assigned to all aromatic carbon atoms (CI)

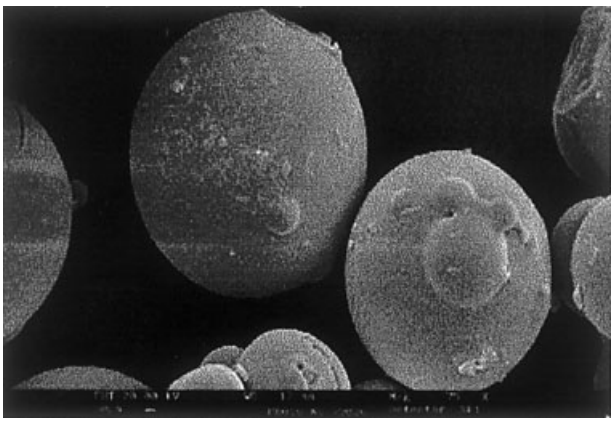


Figure 1 Scanning electron micrograph (SEM) of HDODA-PS (accelerating voltage, 200 kV; magnification, ×100).

except the one that is linked to the backbone chain. The value at 147.887 ppm (small) was attributed to the benzene carbon that is linked to the backbone chain (ipsocarbon atom, C2). The ester carbonyl carbon (C6) appeared at 172.614 ppm. The value at 42.936 is due to C3 carbon. Because this peak was somewhat broad (35–55 ppm), the peak due to methylene of the backbone (which was expected to appear at 30–32 ppm) was not separately visible. The value at 86.693 ppm was assigned to the carbon at the junction of the PS backbone and crosslinker (C5). IR and ^{13}C NMR give a clear picture of the chemical nature of the HDODA–PS system.

Swelling characteristics

The efficacy of the SPPS is largely dependent on the swelling and solvation behavior of the resin. SPPS involves heterogeneous reactions in which solvents of different polarity are used. Hence, one of the essential conditions for a resin to be used in SPPS is that it should be compatible with all on most solvents used in SPPS. The swelling characteristics of HDODA-PS

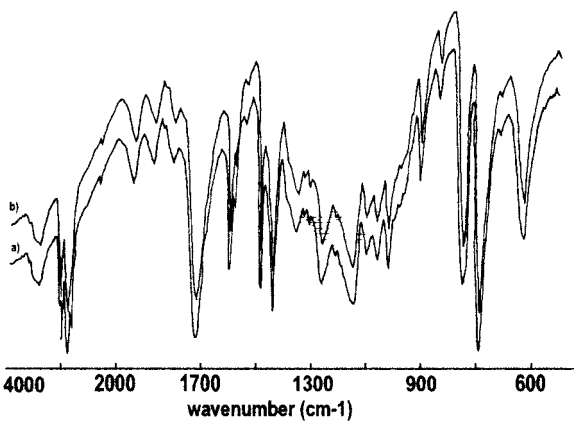


Figure 2 IR spectra of (a) HDODA–PS and (b) TFA-treated HDODA–PS.

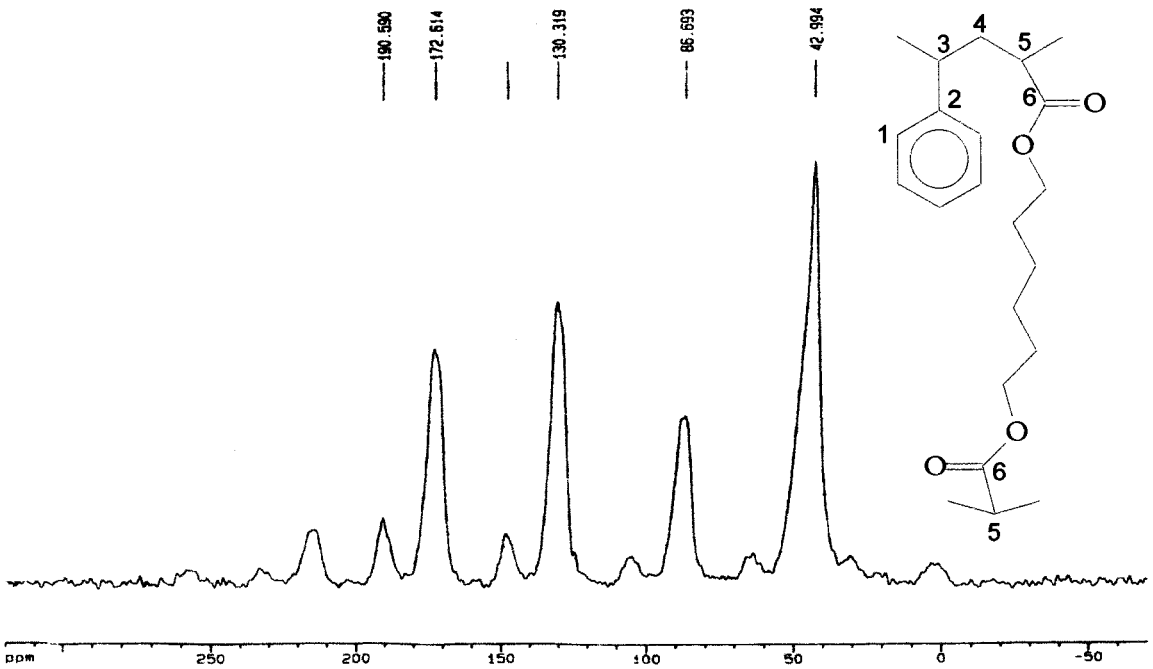


Figure 3 ^{13}C NMR (CP-MAS) spectrum of HDODA–PS.

and DVB-PS are shown in Table I. The new resin exhibits better solvation compared with the DVB-PS resins. The values of those solvents, which are frequently used in peptide synthesis, are given here although similar patterns were observed with other solvents and solvent mixtures. HDODA–PS resins with different crosslink densities exhibit varying affinity towards solvents. As crosslink density increased, the swelling characteristics of the crosslinked resin decreased drastically (Figure 4). However, 1 and 2% crosslinked resin showed almost the same swelling capacity.

Stability of the resin

A stringent conditions for choosing a resin for SPPS is that the resin should be chemically stable until the peptide is separated at the end of the synthesis. If any break of the crosslinker occurs during the cleaving stage, some linear polymer will be formed along with the peptide, resulting in a separation problem. Similarly, if the crosslinker is prone to cleavage during the peptide synthesis, the integrity of the resin will be lost. The chemical inertness of the resin was tested by treating it with neat TFA at 40°C for 48 h. The TFA solution did not show any mass. The stability of the resin was further confirmed from the identical IR spectra of the TFA-treated and untreated resins (Figure 2). The reason for the chemical stability of the resin is that the chance of acidolysis of alkyl ester of the crosslinker is very weak, compared with the benzyl ester formed between the peptide and the benzene part of the polymer. Scheme 2 shows that the chance of formation of the less stable alkyl cation is poor compared with formation of a stable benzyl cation.

A chloromethyl group was introduced on the benzene ring of the polymer by Friedel-Crafts reaction

TABLE I
Swelling Behavior of DVB–PS and HDODA–PS in Solvents Commonly Used in Solid Phase Peptide Synthesis[a]

Solvent	DVB–PS	HDODA–PS
DCM	5.9	10.8
NMP	6.2	8.6
DMF	3.6	4.0
THF	3.2	7.0
$CHCl_3$	8.0	9.0

[a] The values indicate the volume of solvent imbibed by 1 g of resin at room temperature.

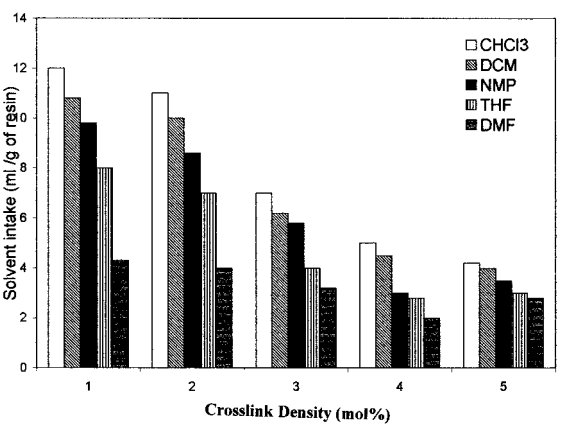
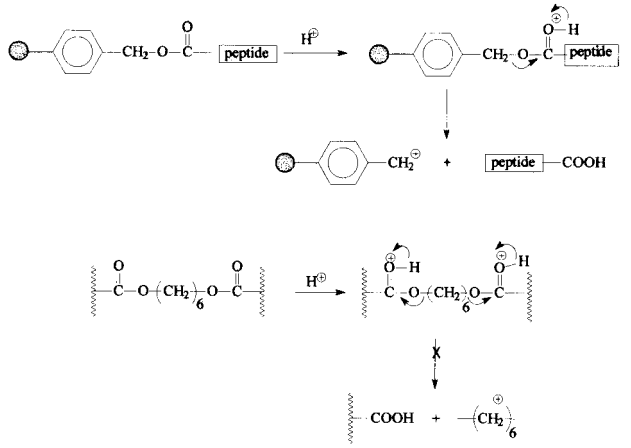


Figure 4 Swelling characteristics of 1–5% HDODA–PS.



Scheme 2 Acidolysis of ester bond in peptide resin and crosslinker of HDODA.

using chloromethyl methyl ether (CMME) and $ZnCl_2$. IR (KBr) analysis is showed peaks at 1420 and 668 cm^{-1} (due to C—Cl; Figure 5). $ZnCl_2$ was suitable for effectively controlling the capacity of the resin. The chlorine capacity, estimated by modified Volhard's procedure,[22] was 2.0 mmol/g.

Syntheses of peptides

The following peptides were synthesized using DVB–PS and HDODA–PS: Peptide 1, VAVAAG (hexapeptide); Peptide 2, QVGQVELG (octapeptide); Peptide 3, Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly; and acyl carrier protein fragment (65–74) (decapeptide). All these peptides are very hydrophobic in nature and are reported as difficult sequences. Peptides 1 and 2 contain bulky side chains and shows high $<SP_\beta>$ values, which is a measure of the peptides' tendency to aggregate.[23] Peptide 3 is the well-known difficult sequence.[24,25] This sequence has been widely accepted as a model peptide to illustrate the synthetic efficiency of a reagent when a polymer or synthetic condition is changed.

Boc Gly was covalently bound to both supports by the cesium salt method. The esterification was estimated by the picric acid method. The amount of first amino acid substitution was kept constant for comparing the supports. The amino capacity of HDODA-PS was 1.9 mmol/g and that of DVB–PS was 1.85 mmol/g. However this difference was achieved by giving more reaction time in the case of DVB–PS. In the case of HDODA–PS, the reaction was completed by 36 h, whereas 48 h had to be used for DVB–PS to obtain the same substitution level. The better swelling features of HDODA–PS allowed it to imbibe more solvents. Hence, the reagent diffused into the interior of the matrix, resulting in faster reaction. In the case of DVB–PS, the steric effect caused by the poor swelling property suppressed the penetration of reagents into the matrix where most of the functional groups reside.

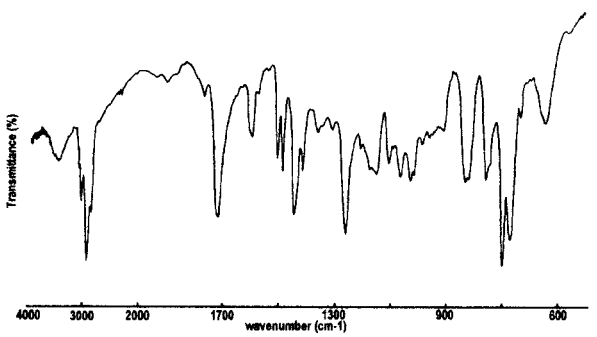


Figure 5 IR spectrum of chloromethylated HDODA–PS.

TABLE II
Protocol Used for the Synthesis of Peptides

Step	Reagent	No × time (min)
1	DCM wash	5 × 2
2	30% TFA in DCM (Boc deprotection)	1 × 30
3	DCM wash	5 × 2
4	5% DIEA in DCM–NMP (1:1 v/v)	1 × 5
5	5% DIEA in NMP	1 × 5
6	NMP	3 × 2
7	2.5 mequiv of active ester of amino acid	1 × 45
	DMSO	1 × 15
	DIEA (3.8 mequiv)	1 × 5
8	Methanol–DCM (33:67, v/v)	5 × 5
9	Kaiser test—if positive repeat step 6-8	
10	DCM wash	5 × 2

The peptides were synthesized using Boc/Bzl strategy. Boc Gly resins (DVB-PS and HDODA-PS) were used for the synthesis of all these peptides. The general protocol for coupling of one amino acid is shown in Table 2.

The coupling of individual amino acids was achieved by the DCC/HOBt active ester method. The coupling was performed in NMP, which can disrupt the aggregation of peptide.[26] DMSO was added at the end of the reaction for the same purpose.[25] In the HOBt active ester method, liberation of HOBt during amidation causes the pH of the medium to rise, resulting in a reduction of reaction rate. The use of DIEA enhances the pH of the medium and thereby increases the reaction rate.

The coupling reactions were monitored by the Kaiser test. In HDODA–PS, most of the coupling was completed by the first coupling itself. A second coupling was, however, given to ensure completion of the reaction. However, more coupling had to be employed in the case of DVB–PS to get a negative Kaiser test.

The peptides were liberated from the supports using neat TFA in the presence of thioanisole and *m*-cresol. The yield of peptide, obtained from the new supports, was higher than that obtained from DVB–

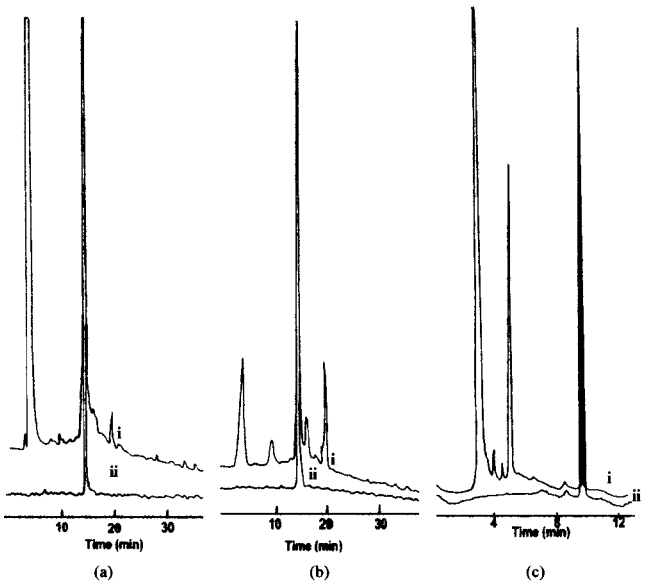


Figure 6 HPLC traces of (a) hexapeptide, (b) octapeptide, and (c) decapeptide obtained from (i) DVB–PS and (ii) HDODA–PS.

PS. The HPLC traces of the crude peptides showed the higher purity of peptide obtained from the new support (Figure 6).

CONCLUSION

The use of HDODA–PS in the synthesis of various peptides has been illustrated. The resin swells extensively in solvents of varying polarity. The yield and purity of peptides obtained when the new resin was used is appreciable in comparison with that of the commercial DVB–PS resin. In conclusion HDODA–PS is an ideal support for the solid-phase synthesis of peptides.

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