CHAPTER 3

SYNTHESIS, FUNCTIONALISATION AND CHARACTERIZATION OF 1,4-BUTANEDIOL DIMETHACRYLATE CROSS-LINKED POLYSTYRENE (PS-BDODMA)
3.1. Introduction

One of the main challenges in peptide synthesis is to establish synthetic routes to homogeneous products of defined covalent structure. Solid phase peptide synthesis is a new synthetic protocol in which a heterogeneous reaction takes place between the solvated resins bound peptide 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 behaviour of the polymer support determines its physicochemical properties.\(^1\) The chemical nature of the monomer, mole percentage of cross-linker, type of diluent, 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.\(^2,3\) The support interacts with reaction medium and has significant role in polymer-supported synthesis of polypeptides. If the support and the substrates are compatible, favourable interaction between the polymer and the substrate molecules occur and improves the rate of the reaction.

Merrifield’s solid-phase technique is widely used in biopolymer synthesis, small molecule organic synthesis and in combinatorial chemistry.\(^4-7\) The DVB-cross linked polystyrene support shows rigidity and high mechanical strength. It swells only in nonpolar solvents because of its high hydrophobic macromolecular network.\(^8,9\) The hydrophilic peptide synthesis on the PS-DVB resin is not so much effective because of its aggregation in nonpolar solvents. The compatibility between the growing peptide chain and the polymer support can be increased by introducing polar polyacrylamide type supports.\(^5,10\) These resins showed effective swelling in polar solvents but their swelling in nonpolar solvents is rather poor. The mechanical stability of these resins is very much less compared to polystyrene based resins. 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 functionalisation the physical and chemical properties can be varied. Solid support with optimal properties has been obtained by radical polymerization of 1,4-butanediol dimethacrylate (BDODMA) with styrene. The support
is cost-effective since they are prepared by an easy single step polymerization using readily available low cost monomers. The functional 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 PS-BDODMA support has been successfully used for the polypeptide synthesis. The anchoring of functional groups and their reactivity were highly enhanced due to the greater chain mobility of BDODMA cross-link compared to rigid hydrophobic PS-DVB support. The attachment of C-terminal amino acid to different functionalised PS-BDODMA resins at regular time intervals were optimized. The time dependent Fmoc and Boc-amino deprotection and coupling of different amino acids were determined and compared with Merrifield resin. The temperature dependence in coupling of different amino acids is also optimized.

3.2. Results and Discussion

3.2.a. Synthesis of BDODMA-cross linked polystyrene (PS-BDODMA) support

The important parameters, which determine the physicochemical properties that render a polymer support favourable 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 provides the desired mechanical integrity and polarity to the resin. PS-BDODMA 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 peptide.

In the initial phase of this work bulk polymerization of BDODMA and styrene were carried out. The polymer obtained after polymerization was grounded and sieved to particles of below 500 mesh size. But this process resulted in particles with irregular shape and after functionalisation with CMME, the resin become 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
Scheme 3.1. Preparation of BDODMA-cross linked polystyrene resin

physical form of the beaded product which lends itself to further conversions.\textsuperscript{11,12} 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 3.1). The insoluble polymer support was obtained as spherical uniform beads. The beads were sieved and the main fractions

Fig. 3.1. SEM of PS-BDODMA polymer support
obtained were of 100-200 mesh size. 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 micrograph (SEM) of the polymer showed that they are uniform spherical beads and the surface is even and smooth (Fig. 3.1).

Table 3.1. Preparation of PS-BDODMA resin by suspension polymerization

<table>
<thead>
<tr>
<th>Mole % of BDODMA in feed</th>
<th>Amount of styrene (g)</th>
<th>Amount of BDODMA (g)</th>
<th>Yield of polymer (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.41</td>
<td>0.23</td>
<td>7.60</td>
</tr>
<tr>
<td>2</td>
<td>10.20</td>
<td>0.45</td>
<td>8.34</td>
</tr>
<tr>
<td>3</td>
<td>10.09</td>
<td>0.68</td>
<td>8.72</td>
</tr>
<tr>
<td>4</td>
<td>9.99</td>
<td>0.91</td>
<td>9.26</td>
</tr>
<tr>
<td>6</td>
<td>9.79</td>
<td>1.36</td>
<td>10.55</td>
</tr>
<tr>
<td>8</td>
<td>9.58</td>
<td>1.81</td>
<td>10.87</td>
</tr>
<tr>
<td>10</td>
<td>9.37</td>
<td>2.26</td>
<td>11.25</td>
</tr>
</tbody>
</table>

3.2.b. Characterization of PS-BDODMA support

The UV and conventional NMR techniques are not suitable for the characterization of the cross-linked polymer support because of its insolubility in all types of solvents. IR spectroscopic method is the apt choice for studying the polymer supported reactions, structural identification, polymeric transformation and functional group identification. IR method is also used to study the extent of transformation in polymer-supported reaction, determination of chlorine capacity and conformational analysis of cross-linked resin bound oligo-leucines in the swollen state.\textsuperscript{3,13,14}
IR and solid-state $^{13}$C CP-MAS NMR spectroscopy is used for the characterization of PS-BDODMA resin. The resin was powdered and pelleted with KBr. IR spectrum gave a sharp and intense peak at 1720 cm$^{-1}$ corresponding to ester carbonyl group of the cross-linker in addition to the peaks of polystyrene (Fig.3.2a). The $^{13}$C CP-MAS solid-state NMR spectroscopic measurements were conducted on a Bruker 300 MSL CP-MAS instrument operating at 75.47 MHz. The spectra were run with fine powder of polymer beads at room temperature and KelF rotor was employed for MAS. The samples were rotated with spectral width of 25000 Hz, the cross polarization time was 22 min and the number of scans was within the range of 200-300. Each sample was rotated with two different spin routes, and by comparing the resultant spectra, the spinning side bands were eliminated. Solid-state $^{13}$C CP-MAS NMR spectra of PS-BDODMA resin showed an intense peak at 130.48 ppm corresponding to aromatic polystyrene carbons and a small peak at 145.30 ppm arising from C-3 carbon of the styrene. The backbone methylene carbon of the polymer gave a singlet at 42.78 ppm and methylenes of the cross-linking agent appeared as a small peak at 67.47 ppm (Fig.3-2b).
3.2.c. Swelling and stability studies of polymer support

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. The extent of swelling of the resin was a measure of its solvation by a given solvent. 1% PS-DVB resin showed excellent swelling behaviour and was considered to be the best support for SPPS. The swelling efficiency of the resin decreases with increase in cross-linking density because of the increased rigidity.

The solvent imbibition of the various cross-linked PS-BDODMA 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 of the bead. Though the 1% PS-BDODMA 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 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 supports for SPPS. These resins are rigid and their swelling characteristics decrease with increase in cross-linking density. 2%, 3% and

Fig.3-3. Swelling comparison of PS-BDODMA resin with various cross-linking densities in different solvents.
4% PS-BDODMA resins are found to be good supports for the synthesis. Since 2% cross-linked support showed better swelling characteristics compared to 3% and 4% cross-linked supports in different solvents, it was selected for the synthesis of peptides. The 2% PS-BDODMA resin showed extensive swelling in various solvents compared to 1% PS-DVB resin and PS-BDODMA 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 Fig.3-3. The chloromethyl PS-BDODMA resin also showed comparable swelling characteristics to that of PS-BDODMA resin, confirming the absence of methylene bridges between adjacent aromatic rings (Fig.3-4). The results showed that the PS-BDODMA resin has superior swelling characteristics in various polar and nonpolar solvents. This can facilitate the diffusion of soluble reactants and reagents into the polymer matrix and increases the rate of the reaction.

The chemical stability of the resin in various solvents and reagents is one of the factors that determine the efficiency of the support in polypeptide synthesis. 2% PS-BDODMA resin was found to be stable enough to withstand various chemistries performed over the resin. The support showed comparable physical and mechanical properties to that of PS-DVB support, permitting identical manipulations such as shaking.
Fig. 3-5. IR (KBr) spectra of PS-BDODMA: (a) original; after 48 h treatment with (b) neat TFA; (c) 20% piperidine DMF; (d) 2 M aqueous NaOH; (e) 2 M NH₂OH in aqueous MeOH; (f) liquor ammonia

and filtration when used in SPPS. The stability of the new resin is tested in commonly encountered peptide synthetic conditions. After 48 h treatment of the resin with 30% TFA/DCM mixture (used for the Boc-removal) did not show any change in the IR spectrum of the resin. Similarly piperidine-DMF (1:4) treatment of the resin (used for the Fmoc-removal) did not show any change in IR spectrum even after 48 h. When the resin
was treated with neat TFA for 48 h, it did not show any weight loss and change in the IR spectrum. The ester group that is present in the resin was stable enough to withstand the nucleophilic cleavage by stronger bases than piperidine like 2 M aqueous NaOH, 2 M NH₂OH in aqueous MeOH and liquor ammonia. After 48 h treatment with the above reagents, IR spectrum of the resin showed no change (Fig.3-5).

3.2.d. Functionalisation of PS-BDODMA resin

1. Chloromethylation

The chloromethylation of polystyrene based resins can be achieved by two methods. In one method HCl gas is passed through a solution of formaldehyde containing Lewis acid (ZnCl₂, H₂SO₄) in which the polymer was suspended and in the second method the swelled resin in DCM was treated with chloromethyl methyl ether (CMME) in presence of Lewis acid such as AlCl₃, ZnCl₂ or SnCl₄. CMME is a good swelling agent for polymer and chloromethylation proceeds easily and there by higher percentage of functionalisation is possible. The vigorous treatment of the polymer with CMME can result additional cross-linking that increases rigidity of the support. The chloromethylation under mild conditions can eliminate this problem. IR spectral studies of PS-DVB resin showed 90% of the chloromethyl group in the resin is introduced at the para-position of the ring whereas ¹³C NMR studies showed that 99% of chloromethylation takes place at para-position. The solvent imbibition studies showed that the polymer swelling is maximum in solvents like DMF, NMP and DCM. In the gel phase, cross-link network of the polymer becomes extremely distant, porosity increases, mechanical resistance to the reagents due to the polymer network decreases and thus allow the reagents to reach the reaction site easily.

Chloromethylation of PS-BDODMA resin can be achieved by a Friedel-Crafts type electrophilic substitution reaction of the aromatic ring. The reagents used for the functionalisation are CMME in presence of a Lewis acid. CMME can be prepared by treating dry HCl with a mixture of methanol and formaldehyde at 0 °C. Anhydrous AlCl₃ is not used as a catalyst for chloromethylation because it forms a complex with the polymer and cannot be washed off completely with common solvents. Anhydrous SnCl₄ has a very strong catalytic activity, the reaction become very fast and can be controlled
by cooling and efficient mixing. For controlled chloromethylation to obtain resin with desired chlorine capacity, anhydrous ZnCl₂/THF is used as the catalyst (Scheme 3.2).

![Graphs](image)

**Fig.3-6.** (a) Time-dependent chloromethylation of PS-BDODMA resin at 55 °C (b) Temperature-dependent chloromethylation of PS-BDODMA resin.

The resin with desired chlorine capacity can be obtained by varying the amount of CMME, catalyst, temperature and duration of the reaction (Fig.3-6 a & b). The scanning electron micrograph of the chloromethyl resin is given in Fig. 3-7. Functionalisation resulted in some morphological changes in the spherical bead. The degree of chloromethylation can be determined by Volhardt's method. No additional cross-linking was observed during the chloromethylation. The swelling characteristics of the chloromethyl resin in different solvents are given in Fig.3-4.
Scheme 3-2. Chloromethylation of PS-BDODMA using ZnCl₂/THF catalyst (1g resin swelled in DCM (20 mL), 0.2mL 1M ZnCl₂/THF and 6mL CMME) (Fig. 3.6.a & b)

Fig.3-7. Scanning electron micrograph of chloromethyl PS-BDODMA resin.

IR and $^{13}$C CP-MAS NMR (solid-state) spectroscopic methods were used for the characterization of chloromethyl PS-BDODMA resin. IR spectrum gave a band at 670 cm$^{-1}$ and 1420 cm$^{-1}$ for C-Cl stretching and a sharp peak at 1250 cm$^{-1}$ corresponds to H-C-Cl bending vibration along with usual band of PS-BDODMA resin (Fig.3-8a). $^{13}$C CP-MAS NMR spectrum of PS-BDODMA resin (Fig.3-8b) showed an intense peak at 130.48 ppm corresponding to aromatic ring. The backbone methylene carbon of polymer observed as a single peak at 42.78 ppm. The methylene carbon of the crosslinker observed as a small peak at 67.47 ppm. The peak at 48.30 ppm is due to the methylene carbon atom of chloromethyl group and a small peak at 135.6 ppm corresponds to C-6 carbon of polystyrene ring.
2. Hydroxymethylation

The hydroxymethyl PS-BDODMA resin can be obtained from the corresponding chloromethyl resin. The swollen chloromethyl resin in methyl cellosolve was refluxed with potassium acetate for 24 h. The acetoxy derivative of the resin formed was converted to hydroxymethyl resin by treating with hydrazine hydrate in NMP. The extent of attachment of hydroxyl group can be estimated by measuring the remaining chlorine content in the resin using Volhardt's method. The chlorine estimation indicates the complete conversion of chloromethyl group to its hydroxyl derivative.

IR and $^{13}$C CP-MAS NMR (solid state) spectroscopic methods were used for the characterization of hydroxymethyl PS-BDODMA resin. IR spectrum gave a sharp peak at 3400 cm$^{-1}$ corresponding to the O-H stretching along with the normal bands of PS-BDODMA resin (Fig.3.9a). $^{13}$C CP-MAS NMR spectrum of PS-BDODMA resin (Fig 3.9b) showed a sharp peak at 129.32 ppm corresponding to aromatic ring. The backbone methylene carbon of the polymer observed as a single peak at 42.92 ppm. The methylene carbon of the cross-linker observed as a small peak at 64.82 ppm. The peak at 62.21 ppm corresponding to the methylene carbon of hydroxymethyl group.
3. Aminomethylation

The aminomethyl PS-BDODMA resin was obtained from its chloromethyl derivative. The pre-swollen chloromethyl resin in NMP was refluxed with potassium phthalimide for 12 h. The phthalimidomethyl resin formed was converted to amino methyl derivative by hydrazinolysis. The extent of conversion was estimated by picric acid titration method.

IR and $^{13}$C CP-MAS NMR (solid state) spectroscopic methods were used for the characterization of aminomethyl PS-BDODMA resin. IR spectrum gave a broad peak at 3450 cm$^{-1}$ and a sharp peak at 1520 cm$^{-1}$ corresponding to the NH$_2$ group along with the usual bands of PS-BDODMA resin (Fig.3-10a). $^{13}$C CP-MAS NMR spectrum of aminomethyl PS-BDODMA (Fig.3-10b) showed a peak at 129.21 ppm corresponding to aromatic ring. The backbone methylene carbon of the polymer observed as a single peak at 42.20 ppm and the methylene carbon of the cross-linker corresponding to the small peak at 64.83 ppm. The peak at 58.93 ppm corresponding to the methylene carbon bearing the amino group. The C-6 carbon of the polystyrene ring observed as a single peak at 136.42 ppm.
3.2.e. C-terminal amino acid incorporation to PS-BDODMA resin

Two methods are widely used for the incorporation of C-terminal amino acid to the chloromethyl resin—triethyl amine method introduced by Merrifield and cesium salt method. In triethyl amine method, chloromethyl resin was refluxed with triethyl amine for 24 h using ethyl acetate as solvent. Quaternary ammonium salt formed on the resin will act as an ion exchange resin that hinders the progress of peptide synthesis. Cesium salt method is the most appropriate method for the attachment of the C-terminal amino acid to the resin. In this method Boc-amino acid was dissolved is minimum volume of water or 4:1 ethanol/water mixture and the solution was neutralized by the addition of saturated solution of $\text{CS}_2\text{CO}_3$ in water. Neutralization is the crucial step that determines the 100% incorporation of amino acid to the resin. The percentage incorporation of C-terminal amino acid was determined after the removal of Boc-protection by picric acid method. The rate of attachment of the cesium salt of Boc-amino acids to the PS-BDODMA resin was optimized by using Boc-Val, Boc-Gly and Boc-Ala as examples. In each reaction, 2 mmol excess of the cesium salt of respective Boc-amino acid was used for the esterification reaction under identical conditions. The results were
compared with PS-DVB resin (Fig.3-11). The results showed that for the PS-BDODMA resin 100% incorporation has taken place in 8 h whereas the PS-DVB resin required 24 h for the quantitative incorporation. This discrepancy appears to be due to the flexible, hydrophilic BDODMA cross-linker of the resin that can allow free interaction between the reactive centers on the support and the respective amino acids and reagents in DMF.

![Graphs showing percentage incorporation over time for PS-BDODMA and PS-DVB resins.](a) (b) (c)

*Fig.3-11. Time-dependent incorporation of C-terminal amino acids (a) Boc-Val; (b) Boc-Ala; (c) Boc-Gly*

The time-dependent attachment of C-terminal Fmoc-amino acids to PS-BDODMA resin was optimized by monitoring the percentage incorporation of Fmoc-Val, Fmoc-Ala and Fmoc-Gly using 1-(2-Mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT). In this method the C-terminal amino acid was attached to the resin via an ester bond using Fmoc-amino acid (2 equiv), MSNT (2 equiv) dissolved in dry DCM in presence of a base N-methyl imidazole (1.5 equiv). The results obtained were compared with PS-DVB resin (Fig.3-12). PS-BDODMA-HMPA resin required 20 min for the quantitative reaction whereas PS-DVB-HMPA resin required 38 min under the same synthetic conditions. Hydroxymethyl derivatives of PS-BDODMA and PS-DVB resins required 24 and 45 min respectively for optimum reaction. The higher rate of incorporation of C-terminal amino acids for HMPA functionalised resins could be due to the spacer effect that enables the functional groups to project away from the macromolecular environment of the polymer.
3.2.f. Time-dependent cleavage of Boc-amino protection

The rate of Boc-deprotection of the $N_a$-amino group of the first amino acid attached PS-BDODMA resin was optimized and compared with Merrifield resin (Fig 3-13a). Boc-Val attached resin was used for the optimization reaction. The Boc-removal was achieved by treating the resin with 30% TFA/DCM from the amino acid attached PS-BDODMA resin. The rate of Boc-removal was determined by measuring the free amino group of the resin at different time intervals using the picric acid method. PS-BDODMA resin required 14 min whereas PS-DVB resin required 20 min for quantitative removal of Boc-group. These results showed that the reaction rate in PS-BDODMA resin is approximately twice that in the PS-DVB resin.

Fig.3-12: Time-dependent incorporation of C-terminal amino acids (a) Fmoc-Val (b) Fmoc-Ala, (c) Fmoc-Gly.
Fig. 3-13. (a) Time dependent Boc-removal of C-terminal Boc-Val from various supports using 30% TFA in DCM (b) Time-dependent Fmoc-removal from C-terminal Fmoc-Val attached PS-BDODMA-HMPA resin with various concentration of piperidine in DMF (c) Time dependent Fmoc removal C-terminal Fmoc-Val from various supports using 20% piperidine in DMF

3.2.g. Time-dependent cleavage of Fmoc-amino protection

The rate of removal of N$_\alpha$-Fmoc protection in first amino acid attached PS-BDODMA resin was optimized and compared with that of Merrifield resin. Fmoc-Val attached resin was used for the optimization reaction. A time-dependent study of Fmoc-removal using 5%, 10%, 15%, 20% & 25% piperidine in DMF from the C-terminal amino acid of PS-BDODMA-HMPA resin showed that the percentage cleavage in unit time increases with base concentration (Fig. 3-13b). The time difference for quantitative Fmoc-removal was negligible when compared with the time dependent cleavage using 20% and 25% base concentrations. A 20% piperidine/DMF was therefore used as the N$_\alpha$-deprotection reagent throughout the synthesis to avoid any side reactions and racemisation at higher base concentrations (Fig. 3.13c). The rate of cleavage was determined by measuring the optical density of the dibenzofulvene-piperidine adducts. The PS-BDODMA resin required 14 min whereas the PS-BDODMA-HMPA resin required only 9 min for quantitative removal. PS-DVB resin required 30 min for 100% Fmoc-removal whereas PS-DVB-HMPA resin
required only 15 min. The results showed that the reaction rate in PS-BDODMA resin is twice that in the PS-DVB resin. The higher solvation and swelling characteristics of this resin in the reaction medium may enhance the free interaction of the protected amino acids and cleavage reagent improving the reaction rate.

3.2.h. Temperature-dependence in the rate of amino acid coupling in SPPS

The coupling efficiencies may be increased in a temperature dependent manner because of thermal disruption of inter-chain aggregates. In order to study the temperature dependence in coupling reaction, a model peptide leucyl-alanyl-glycyl-valine is synthesized by using Fmoc-amino acids. A comparative time-dependent acylation at different temperatures were carried out on PS-BDODMA, PS-BDODMA-HMPA and PS-DVB resins to find out the most efficient support for peptide synthesis. The coupling efficiencies at 30 °C, 40 °C and 50 °C were measured at every 5 min interval. Among the resins the extent of reaction in the first 10 min was higher for PS-BDODMA-HMPA and showed about 10% increase in coupling efficiency compared to about 7% increase in PS-BDODMA and 5% in PS-DVB resins for every 10°C rise in temperature (Fig3.14). The HPLC analysis of the peptides showed that the peptide synthesized at lower temperature is comparatively pure than that synthesized at elevated temperatures.

![Graphs](Fig.3-14a. Temperature-dependent coupling of Fmoc-Gly)
Fig. 3-14 b. Temperature-dependent coupling of Fmoc-Ala

Fig. 3-14 c. Temperature-dependent coupling of Fmoc-Leu
3.2.i. Time and temperature-dependent cleavage of peptides

In order to optimize the temperature dependence in the rate of cleavage of peptide from the resin, Leu-Ala-Gly-Val attached PS-BDODMA-HMPA and PS-BDODMA resins were taken as examples. The resin was suspended in TFA for the cleavage of the peptide. The rate of cleavage is measured at different temperature in regular time intervals. The comparative time-dependent removal of the peptide at room temperature (30 °C) showed 97% cleavage in 2 h from PS-BDODMA-HMPA resin whereas PS-BDODMA resin required 18 h for 94% cleavage and PS-DVB resin required 20 h for 85% cleavage (Fig.3-15a). The peptide cleavage reaction at 40 °C gave 94% yield in 1 h from PS-BDODMA-HMPA resin whereas PS-BDODMA resin required 8 h for 92% cleavage and PS-DVB resin required 12 h for 83% cleavage (Fig 3-15 b). The HPLC analysis showed that the peptide cleaved at 30 °C is comparatively pure than that obtained at elevated temperatures. Though the rate of cleavage increases with temperature, the purity of peptides decreases. This is because of the denaturation of peptides at higher temperatures.

**Fig.3-15:** Time-dependent cleavage of tetra peptide (Leu-Ala-Gly-Val) from different resins (a) cleavage at 30 °C (b) cleavage at 40 °C.
3.3. Experimental

3.3.a. Materials and methods

1,4-Butanediol dimethacrylate, styrene, poly(vinyl alcohol) (MW~75 000), benzoyl peroxide, hydrazine hydrate, potassium phthalimide, potassium acetate and methyl cellosolve were obtained from Aldrich chemical company USA. Boc-amino acids, diisopropylethyl amine, piperidine were purchased from Sigma chemicals company, USA. 4-Dimethyl amino pyridine (DMAP), dicyclohexyl carbodiimide (DCC), 2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyl uroniumhexafluorophosphate (HBTU), 4-hydroxy methylphenoxyacetic acid, chloromethyl, aminomethyl and hydroxymethyl PS-DVB resins, 1-(2-mesitylenesulfonfyl)-3-nitro-1,2,4-triazole (MSNT), N-methyl imidazole and Boc and Fmoc amino acids were purchased from Novabiochem Ltd, U.K. Chloromethyl methyl ether (CMME) was prepared according to literature procedure. All solvents used were obtained from E. Merck (India), and SISCO chemicals (Bombay). All solvents were purified according to literature procedure before use. BDODMA and styrene were distilled under vacuum before use.

IR spectra were recorded on a Shimadzu IR 470 spectrophotometer in KBr pellets. The $^{13}$C-cross polarization magic angle spinning (CP-MAS) solid-state NMR measurements were conducted on a Brucker 300 MSL CP-MAS instrument operating at 75.47 MHz. The spectra were run with a fine powder of polymer beads at room temperature. Scanning electron micrographs were recorded on a JSM Joel-35 scanning electron microscope.

3.3.b. Preparation of chloromethyl methyl ether

To a 1L round-bottomed flask fitted with a glass tube reaching nearly to the bottom of the flask, methanol (66 mL) and formaldehyde (126 mL) were added. The mixture was cooled to 0 °C and a stream of dry HCl were allowed to pass through it. After 2 h, the reaction mixture become turbid and chloromethyl methyl ether separated out. The layer of CMME was separated and kept over anhydrous calcium chloride below room temperature.
3.3.c. Preparation of PS-BDODMA support by suspension polymerization

Styrene was washed with 1% NaOH solution and then with distilled water to remove the inhibitors and dried over anhydrous Na$_2$SO$_4$. In a typical experiment, a four-necked reaction vessel equipped with a thermostat, teflon stirrer, water condenser and nitrogen inlet and a dropping funnel were used. A 1% solution of poly(vinyl alcohol) (MW~75 000) was prepared by dissolving PVA (1.1 g) in doubly distilled water (110 mL) at 80 °C. A mixture of styrene, BDODMA and benzoyl peroxide (0.5 g) dissolved in toluene (10 mL) were added to PVA solution 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 is allowed to continue for 6 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): 1720, 1480 cm$^{-1}$(ester), 755, 700 cm$^{-1}$ (aromatic).

3.3.d. 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 was 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.

3.3.e. 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$_2$OH in aqueous methanol (10 mL) and liquor ammonia (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 thoroughly with ethanol (3 x 50 mL), water (3 x 50 mL), acetone (3 x 50 mL), DCM (3 x 50 mL), dioxane (3 x 50 mL) and ether (3 x 50 mL), dried, weighed (100 mg) and IR (KBr) spectra of these resins were compared with the original.

3.3.f. Functionalisation of PS-BDODMA resin

1. Chloromethylation

a. Preparation of 1 M ZnCl₂ solution in dry THF

ZnCl₂ (2 g) was mixed in few drops of conc. HCl, add distilled water and heated over a flame till the solid dissolves. It was then heated vigorously to evaporate water and HCl and then stirred with a glass rod in vacuum to get a white powder. The anhydrous ZnCl₂ (1.5 g) was dissolved in dry THF (10 mL) to form 1M ZnCl₂ solution.

b. Functionalisation

Chloromethyl functional group was introduced into the resin by treating with chloromethyl methyl ether in presence of anhydrous ZnCl₂. 2% PS-BDODMA resin (5 g) was swelled in DCM in a dry two-necked round bottomed-flask. After 1 h, the excess DCM was filtered off. The chloromethyl methyl ether (30 mL) along with the catalyst (ZnCl₂/THF (1 mL) was added to the mixture and kept at 55 °C for 3 h. The mixture was cooled and filtered using a sintered glass funnel and washed with THF (5 x 30 mL), THF/water (1:1, 4 x 30 mL) methanol (5 x 30 mL) and then soxhleted with THF and methanol.

IR (KBr): 1720, 1480 cm⁻¹ (ester), 1250 cm⁻¹ (CH₂-Cl)

c. Estimation of halogen capacity by Volhardt’s method

Halogenated PS-BDODMA resin (50 mg) was treated with pyridine (5 mL) in a Kjeldahl flask for 6 h at 100-110 °C. The resin was removed by filtration, washed with acetic acid/water (1:1, 30 mL). The filtrate and the washings were acidified with conc. HNO₃ (5 mL). A saturated solution of AgNO₃ (0.1 N, 5 mL) was added to the mixture and stirred well. The excess AgNO₃ was determined by back titration with standard ammonium thiocyanate solution (0.1 N) using ferric alum as indicator (modified Volhardt’s method).²⁶ A blank was also performed and the halogen capacity calculated from the titre values.
2. Hydroxymethylation

Hydroxymethy resin was obtained from its chloro derivative. The chloromethyl 2% PS-BDODMA resin (2 g, 0.68 mmol/g) was suspended in methyl cellosolve (12 mL). Potassium acetate (1.34 g, 13.6 mmol) was added and the reaction mixture was stirred at 130 °C for 48 h. The resin was filtered and washed with NMP (5 × 25 mL), DCM (5 × 25 mL), ethanol (5 × 25 mL) and methanol (5 × 25 mL). The acetoxy resin formed was converted to its hydroxyl derivative by stirring the resin with hydrazine hydrate (99%, 0.66 mL, 13.6 mmol) in NMP (6 mL) for about 72 h at room temperature.

Estimation of hydroxyl capacity:

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 (0.1 N) NaOH. Capacity was observed as 0.69 mmol/g.

3. Aminomethylation

Aminomethyl resin was prepared from chloromethyl resin. The chloromethyl 2% PS-BDODMA resin (2 g, 0.68 mmol/g) was suspended in NMP (20 mL). Potassium phthalimide (2.52 g, 13.6 mmol) was added and the reaction mixture was stirred at 120 °C for 12 h. The resin filtered and washed with NMP (5 × 30 mL), dioxane (5 × 30 mL), ethanol (5 × 30 mL) and methanol (5 × 30 mL). The dried resin was suspended in ethanol (20 mL) and hydrazine hydrate (99%, 0.66 mL, 13.6 mmol) was added. The reaction mixture was refluxed for 8 h. The resin was collected by filtration and washed with hot ethanol and methanol. The product resin was dried in vacuum (1.97 g).

Estimation of amino group-picric acid method

The aminomethyl resin (2 mg) was taken a sintered Gisin’s tube and treated with 0.1 M picric acid (2 × 5 mL × 5 min). The resin was washed thoroughly with DCM to remove the unbound picric acid. The resin bound picric acid was eluted with 5% DIEA in DCM till the eluate was clear. The eluate was made up to a definite volume (15 mL) using 95% ethanol. A definite volume of this solution (0.5 mL) was diluted to 5 mL with
95% ethanol. The optical density of this solution was measured at 358 nm. The substitution level of the first amino acid can be calculated from the OD value, weight of the resin taken and the extinction coefficient of picrate (ε\textsubscript{358} = 14,500). Amino capacity = 0.69 mmol/g.

3.3.g. Preparation of PS-BDODMA-HMPA resin

4-Hydroxymethylphenoxyacetic acid (364 mg, 2 mmol), HBTU (794 mg, 2 mmol), HOBt (270 mg, 2 mmol), DIEA (250 μL, 2 mmol) were added to pre-swollen aminomethyl resin (1 g, 0.69 mmol) in DMF and the reaction mixture was kept at room temperature for 1 h with occasional shaking. The quantitative conversion was estimated by ninhydrin test. The resin was filtered and washed with DMF (3 x 30 mL), dioxane/H\textsubscript{2}O (1:1, 3 x 30 mL), MeOH (3 x 30 mL) and ether (3 x 30 mL). The resin was collected and dried in vacuum. Hydroxyl capacity = 0.66 mmol/g. IR(KBr): 3400 cm\textsuperscript{-1} (NH), 3380 cm\textsuperscript{-1} (OH), 1643 cm\textsuperscript{-1} (NHCO).

3.3.h. Preparation of PS-DVB-HMPA resin

4-Hydroxymethylphenoxyacetic acid (346 mg, 1.9 mmol), HBTU (720 mg, 1.9 mmol), HOBt (270 mg, 1.9 mmol) and DIEA (240 μL, 1.9 mmol), were added to pre-swollen aminomethyl resin (1 g, 0.65 mmol) in DMF. The reaction mixture was kept at room temperature for 1 h with occasional shaking. A second coupling reaction was conducted for quantitative conversion. The resin was filtered and washed with DMF (3 x 30 mL), dioxane/water (1:1, 3 x 30 mL), MeOH (3 x 30 mL) and ether (3 x 30 mL). The resin was collected and dried in vacuum. Hydroxyl capacity = 0.62 mmol/g.

3.3.i. Esterification of Boc-amino acid to chloromethyl resin

The C-terminal amino acid in the target peptide sequence was attached to the resin by cesium salt method. 2.5 mmol of Boc-amino acid was dissolved in ethanol (10 mL) and was treated with a saturated solution of Cs\textsubscript{2}CO\textsubscript{3} till the pH become 7. Ethanol was evaporated under vacuum. The water present in the solution was removed by azeotropic distillation with benzene till a dry white powdery cesium salt of Boc-amino acid was obtained. The cesium salt was dissolved in dry NMP (1 mL) and added to a pre-swollen chloromethyl resin in NMP. The suspension was kept at 50 °C for 24 h with
occasional shaking. The resin was filtered and washed with NMP (6 x 30 mL), NMP:water (1:1, 6 x 30 mL), DCM (6 x 30 mL), methanol (6 x 30 mL) and ether (6 x 30 mL) and dried in vacuum.

3.3.j. Time-dependent esterification of Boc-amino acids to PS-BDODMA and PS-DVB resins

The amino acids were attached to the resins by cesium salt method as described above. Boc-Val, Boc-Gly and Boc-Ala were attached to the resins as examples. The PS-BDODMA resin (500 mg, 0.34 mmol Cl) and PS-DVB resin (500 mg, 0.33 mmol Cl) were used for the synthesis. The cesium salt of the respective amino acids were added to the swollen resin in NMP and about 5 mg of the resin was withdrawn from the reaction mixture at every 1 h interval up to 24 h and each aliquot was washed thoroughly with NMP, NMP/water, DCM, MeOH and ether. The Boc protection was removed by treating the resin with 30% TFA in DCM for 30 min, washed with DCM (5 x 25 mL) and neutralized with 5% DIEA in DCM. The resin was washed thoroughly with DCM (5 x 25 mL), MeOH (5 x 25 mL) and ether (5 x 25 mL). The dried resin (2 mg) was swollen in DCM and 0.1 M picric acid was added to it and kept for 10 min. The resin was washed with DCM and the adsorbed picrate was eluted by 5% DIEA in DCM. From the OD (358 nm) value of the eluate, the amino capacity of the resin and also the extent of amino acid incorporation can be calculated.

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

General procedure

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 a desired time the reactants were washed off with DCM (6 x 30 mL), DMF (6 x 30 mL), MeOH (5 x 25 mL) and ether (5 x 25 mL). The Fmoc-protection was removed by 20% piperidine/DMF and coupling yield was determined from the UV absorption of dibenzofulvene-piperidine adduct.
3.3.1. **Time-dependent esterification of Fmoc-amino acid to PS-BDODMA, PS-BDODMA-HMPA, PS-DVB and P-DVB-HMPA resins**

The amino acids were attached to the resins by using MSNT coupling method as the procedure described above. Fmoc-Val, Fmoc-Gly and Fmoc-Ala were used for the esterification reaction. Hydroxymethyl PS-BDODMA resin (500 mg, 0.35 mmol OH), PS-BDODMA-HMPA resin (500 mg, 0.34 mmol OH), hydroxymethyl PS-DVB resin (500 mg, 0.32 mmol OH) and PS-DVB-HMPA resin (500 mg, 0.31 mmol OH) were used for the reaction. About 5 mg of the resin was withdrawn from the reaction mixture every 5 min up to 1 h. The resin was washed with DCM (5 x 25 mL), MeOH (5 x 25 mL) and ether (5 x 25 mL). Accurately weighed resin was suspended in 20% piperidine in DMF for 30 min. The resin was filtered off and the OD of the piperidine solution was measured at 290 nm. From the OD the extent of attachment can be calculated.

3.3.m. **Time-dependent Boc deprotection**

The Boc group was removed from the Boc-amino acid attached resin using 30% TFA/DCM solution. About 5 mg of the resin was withdrawn from the reaction mixture in 2 min interval for 30 min and the resin was washed with DCM (5 x 25 mL), MeOH (5 x 25 mL), ether (5 x 25 mL) and dried. Accurately weighed amino acid attached resin was treated with 0.1M picric acid and the amount of free amino group was estimated by measuring the OD of picrate adsorbed on the resin at 358 nm.

3.3.n. **Time-dependent Fmoc-deprotection**

Fmoc-amino acid attached resin (250 mg) was treated with 20% piperidine /DMF (10 mL). About 5 mg of the resin was withdrawn from the reaction mixture at 1 min intervals up to 30 min and the resins were washed with DMF (5 x 10 mL), MeOH (5 x 10 mL), ether (5 x 10 mL) and dried. Accurately weighed resin was treated with 0.1M 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 10 mg of the partially Fmoc cleaved resin in 3 mL 20% piperidine in DMF for 30 min. The percentage cleavage was estimated from the OD of dibenzofulvene-piperidine adduct at 290 nm.
3.3.0. Time-dependent coupling of amino acids at different temperatures

Fmoc protection of the 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 (6 \times 10 mL). Fmoc amino acid (3 equiv relative to the amino capacity), HOBt (5 equiv) and HBTU (3 equiv) in DMF (2 mL) were shaken with the resin. About 5 mg of the resin was withdrawn from the reaction mixture every 5 min up to 1 h. The resin was washed with DMF (6 \times 10 mL), MeOH (6 \times 10 mL), ether (6 \times 10 mL) and dried. The Fmoc content in the resin was determined by measuring the OD of 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 regulated with a rheostat.

3.3.p. Detachment of peptide from the PS-BDODMA support

The cleavage times of the peptide from hydroxymethyl PS-BDODMA and PS-BDODMA-HMPA resins were optimized. 50 mg of the Leu-Ala-Gly-Val-resins were treated separately with TFA (2.85 mL) and water (150 \mu L) for 1 h to 18 h at 30 and 40°C. The yield of peptide was calculated by comparing the weight of the peptidyl resin and the amount of peptide obtained.
References


