CHAPTER 2

Current Trends in SPPS
Current Trends in Solid Phase Peptide Synthesis

SPPS has witnessed a dramatic progress ever since its inception. Various polymer supports, new linkers, protecting groups for amino, carboxylic and side chain functional groups, coupling methods and additives were later introduced which make the solid phase synthesis simple and foolproof.

Polymer Support

Merrifield introduced copoly(styrene-divinylbenzene) as the polymer support for peptide synthesis. Additional polymer supports were explored based on the concept that the insoluble support and peptide chain should be of comparable polarities. Sheppard and co-workers developed a series of polyacrylamide supports, which showed improved solvation in polar solvents.

As these and Merrifield resin were incapable of withstanding pressures in continuous flow method, highly permeable Kieselguhr was successfully used as a matrix with polyacrylamide support. In addition to Kieselguhr, Poly HIPE (High Internal Phase Emulsion), controlled pore glass (CPG), Cellulose and polypropylene membranes were used for synthesis of various biologically active peptides.

In response to the need for large number of peptide analogs in small quantities for antibody screening, interest has been focussed on supports for multiple peptide synthesis. Some of the recently appeared techniques are:
Multi-pin synthesis techniques (PIN)-utilizing acrylic acid-coated polyethylene rods and 2-hydroxymethacrylate grafted polyethylene supports.

Multiple antigen peptide system (MAP)-utilizing lysine branched polystyrene resin core matrix.

Simultaneous Multiple peptide synthesis (SMPS) or Tea Bag Method - utilizing polystyrene in polypropylene mesh packets.

Multi column peptide synthesis - utilizing Macrosorb-SPR resin

Cellulose disc multiple peptide synthesis.

Bayer has used PEG grafted polystyrene for SPPS, making use of PEG's ability to solubilise peptides and to overcome the laborious process associated with liquid phase method in which PEG is used as the soluble support. Mendre et al. used polyacrylamide gel resin (Expansin™) for the synthesis of endothelin and its derivatives. Polyethyleneglycol dimethacrylamide copolymer (PEGA), introduced by Meldal and co-workers, shows greater swelling and solvation, and has been used for the synthesis of difficult sequences -ACP 65-74 segment- and other peptides.

Triethyleneglycol dimethacrylate (TEGDMA) and tetraethyleneglycol diacrylate (TTEGDA) based polystyrene support have been developed in this laboratory for peptide synthesis (Fig 2.1. and 2.2.). These supports possess both the positive features of Merrifield's resin and PEG based system, and unlike PEG grafted polystyrene, these supports can be functionalized in high capacity. Apart from polymer development, the utility of polystyrene and amide supports have been improved by suitable linkers and handles.
Fig. 2.1. TEGDMA-crosslinked polystyrene

Fig. 2.2. TTEGDA crosslinked polystyrene
Protecting Groups

Of the various amino protecting groups, tert-butyloxycarbonyl (Boc) and fluorenylmethoxycarbonyl (Fmoc) are widely used. Boc group can be cleaved under acidic conditions and Fmoc group under basic conditions, hence they are considered as orthogonal protecting groups. For the removal of Boc group, 30% TFA in DCM is used. Though neat TFA is usually used for reducing the time required for the synthesis of long peptides, studies show that incomplete removal of Boc group may happen due to the insufficient swelling of the resin in TFA. Earlier, 4N HCl-dioxane was used for the deprotection. However, this reagent was not sufficient to give complete removal of Boc group. TFA in DCM provides an excellent medium for the maximum solvation of peptide resin.

Fmoc group can be easily and rapidly removed by using a secondary amine (e.g., 10% piperidine in DMF). Unlike Boc method, the deprotection step in Fmoc strategy avoids the neutralization stage, thus reducing the time for synthesis. Besides this, Fmoc group permits the easy monitoring of the progress of the synthesis spectrophotometrically and hence Fmoc amino acids are widely used in continuous flow synthesis. However, recent studies reveal the fact that Fmoc group induces β-sheet conformation, hence in solid phase peptide synthesis, using Fmoc protecting strategy incomplete removal of Fmoc group is frequently noted. The side chain of Boc amino acids are protected by benzyl based groups. Boc/Bzl groups are removed during the cleavage step of peptide from the support. In Fmoc strategy, the side chains are protected by
t-butyl groups which are stable in basic conditions, but are very sensitive to acid treatments.

**Coupling Reagents**

The formation of amide bond is the key step in peptide synthesis. The reagent used for coupling should be able to form the peptide bond in mild conditions, and the reaction should be fast. Above all it should retain the optical integrity of the amino acid. The appearance of several coupling reagents in literature is the reflection of the importance of these compounds in peptide synthesis. Some of the recently introduced coupling reagents are given in Table 2.1.

In spite of all these achievements, dicyclohexyl carbodiimide (DCC) is still the widely used coupling reagent. The mechanism of the reaction of DCC with carboxylic acid via the formation of very reactive O-acyl isourea intermediate has been proposed by Khorana. DCC when used with excess carboxylic acid make symmetrical anhydride which are capable of attacking the nucleophile.

Recently Slebioda investigated the mechanism of carbodiimide-mediated coupling reaction in peptide synthesis and reported that the formation of unstable N-acyl amino anhydride from 5(4H)-oxazolone is responsible for the little epimerisation observed in DCC mediated reaction.

Besides DCC, active esters of amino acids have been widely used in peptide synthesis. As these are stable at room temperature, they can be
prepared and stored for long time for the direct reaction in coupling. Among these, penta fluoro phenyl esters of amino acids are very popular.97

Miyazawa et al. used Cu(II) chloride for suppressing the racemisation in DCC assisted coupling reaction.98 1-Hydroxy benzotriazole (HOBT) is used as coupling additive which can prevent racemisation.99 HOBT is used either in conjunction with DCC or as active ester or built into a stand-alone reagent in the form of phosphonium100,101 or uronium salt.102,103 The presence of tertiary amine e.g. diisopropylethyl amine (DIEA) has been reported104 to increases the reaction rate and product purity of carbodiimide/HOBt mediated coupling. The rate of coupling in this case is comparable with other rapid coupling method using (1-H,1,2,3-benzotriazol-1-yloxy tris(dimethylamino) phosphonium hexafluoro phosphate (BOP) or 2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (TDBTU) (Table 2.1.) and no racemisation is noted.104

Comparative study of various coupling agents to couple hindered peptides show that urethane protected amino acid N-carboxy anhydride (UNCA) and bromo-tris-(pyrrolidino)-phosphonium hexafluorophosphate (PyBroP) are excellent reagent compared to 2-(1-H Benzotriazol-1-yl)1,1,3,3-tetramethyl-uronium hexafluoro phosphate (HBTU), pentafluorophenyl esters or mixed anhydrides.123
Table 2.1. Coupling reagents used in peptide synthesis

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Chemical Structure</th>
<th>Remark</th>
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<tbody>
<tr>
<td>1. (1-H1,2,3benzotriazol)-1-yl-oxy tris(dimethylamino) phosphonium hexafluoro phosphate (BOP)(^{100,105,106})</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>• HMPA is evolved during work up.</td>
</tr>
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<td></td>
<td></td>
<td>• Suitable for difficult couplings.</td>
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<tr>
<td>2. 2-(1-H Benzotriazol-1-yl)1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU)(^{107})</td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>• Toxic tetramethyl urea is formed during work up.</td>
</tr>
<tr>
<td>3. 2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TDBTU)(^{107})</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>• Suppress racemisation effectively.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Danger of side reactions.</td>
</tr>
<tr>
<td>4. 2-(2-oxo-1-(2H)-pyridyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TPTU)(^{107})</td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td>• Useful for segment condensation.</td>
</tr>
<tr>
<td>5. 2-succinimido-1,1,3,3-tetramethyl uronium tetrafluoroborate (TSTU)(^{107})</td>
<td><img src="image5" alt="Chemical Structure" /></td>
<td>• Useful for in situ formation of -Osu and -ONB esters and coupling in aqueous solutions.</td>
</tr>
<tr>
<td>6. 2-(5-norbornene-2,3-dicarboxamido)-1,1,3,3-tetramethyl uronium tetrafluoroborate (TNTU)(^{107})</td>
<td><img src="image6" alt="Chemical Structure" /></td>
<td>• Free aliphatic hydroxyl function are not affected.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Useful for continuous flow peptide synthesis.</td>
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<tr>
<td></td>
<td></td>
<td>• Useful for coupling in aqueous media.</td>
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</table>
| 7. Urethane protected amino acid N-carboxy anhydride (UNCA) | ![Chemical Structure](https://via.placeholder.com/150) | - CO₂ is the only by product.  
- Moisture sensitive. |
|---|---|---|
| R = Side chain of amino acid  
R' = Fmoc, Boc, Z. | | |
| 8. Fmoc-amino acid fluoride | ![Chemical Structure](https://via.placeholder.com/150) | - Stabler than corresponding chloride.  
- Less reactive than chloride |
| | Fmoc-N-C-F | |
| R = Side chain | | |
| 9. Tetrabutyl ammonium hydroxide/ p-toluene sulphonyl chloride | TBA'OH/TsCl | - Low cost, readily available substrate  
- Application in SPPS is limited |
| | | |
| 10. 1-(β-naphthalene sulfonyl oxy)-benzotriazole (NSBT) | ![Chemical Structure](https://via.placeholder.com/150) | - Coupling can be done in alcohol, alcohol-water mixture. |
| | | |
| 11. 1H-(1,2,3-benzotriazol-1-yloxy)-tris(pyrrolidino) phosphonium hexafluoro phosphate (Py BOP) | ![Chemical Structure](https://via.placeholder.com/150) | - More effective than DCC in yield of product and reaction time.  
- Like BOP, stable. |
| | | |
| 12. Bromo-tris-(pyrrolidino)-phosphonium hexafluorophosphate (PyBroP) | ![Chemical Structure](https://via.placeholder.com/150) | -do- |
**Purification and Characterization of Peptides**

Along with the upsurge in the progress of peptide synthesis, there has been a dramatic change in the field of characterization and purification of peptides and proteins. The recent developments in chromatography and mass spectroscopy for purification and characterization of peptides and proteins make peptide chemistry an interesting and challenging field.

<table>
<thead>
<tr>
<th><strong>13. Bromo-tris(dimethylamino)-phosphonium hexafluorophosphate (BroP)</strong>&lt;sup&gt;115&lt;/sup&gt;</th>
<th><img src="image" alt="BroP" /></th>
<th>-do-</th>
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<tbody>
<tr>
<td>Toxic HMPA is one of the by-product.</td>
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<tr>
<th><strong>14. Benzotriazolyloxy-bis-(pyrrolidino)-carbonium hexafluorophosphate (BBC)</strong>&lt;sup&gt;116&lt;/sup&gt;</th>
<th><img src="image" alt="BBC" /></th>
<th>Efficient than BOP and HBTU.</th>
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<tr>
<td>No toxic side products.</td>
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<tr>
<th><strong>15. 3-Dimethyl phosphinothiol-2-(3H)-oxazoline (DMPTO)</strong>&lt;sup&gt;117&lt;/sup&gt;</th>
<th><img src="image" alt="DMPTO" /></th>
<th>Racemisation free coupling reagent for segment condensation and cyclisation.</th>
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<tr>
<th><strong>16. Bis (Boc) amino acid fluoride</strong>&lt;sup&gt;118&lt;/sup&gt;</th>
<th><img src="image" alt="Bis-Boc" /></th>
<th>Like Fmoc acid fluoride.</th>
</tr>
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<tr>
<th><strong>17. Fmoc amino acid chloride</strong>&lt;sup&gt;119-122&lt;/sup&gt;</th>
<th><img src="image" alt="Fmoc" /></th>
<th>Applicable to SPPS by in situ conversion to N-Hydroxy benzotriazol ester in presence of tertiary amine.</th>
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</table>
Gel-filtration is one of the very early, but commonly applied purification method. The separation in gel-filtration is achieved by the difference in the size of molecules, which move at different speeds along the cavity of the stationary phase. Gel-filtration is an efficient method to remove low molecular weight impurities from the crude product obtained after synthesis. However the resolution of the chromatogram is not appreciable in the case of peptides having related size.

With the advent of high performance liquid chromatography (hplc), the scenario has largely changed. HPLC with binary and ternary solvent system can provide excellent resolving power. By the use of very fine stationary phase, and precisely operating pumps, hplc can separate the peptides very quickly. Normal and reversed-phase columns are extensively used in hplc systems. However certain hydrophobic peptides tend to aggregate and can cause deterioration of the column. Liquid chromatography finds less use in the case of severely hydrophobic peptides which are insoluble in solvents.

Some of the other techniques used are ion exchange chromatography, counter current distribution, perfusion chromatography, capillary electrophoresis, reversed phase flash chromatography and high resolution 2D electrophoresis. In fact the characterization and purification in most cases involves the combination of some or all of these techniques.

The determination of structural information for peptides and protein requires the utilization of analytical techniques in a complementary fashion. Amino acid analysis and sequencing are used extensively for the
characterization of peptides. For small peptides, NMR can give best results. However, the structure elucidation of larger peptides would be complex using NMR especially by 1D NMR. Recently, mass spectrometry (MS) with different modes are extensively used for the characterization of large sized peptides. Merrifield et al. used this technique to evaluate the different synthetic protocol. Based on the identification and quantitation of peptide byproducts, they could measure the levels of deletion and insertion peptides by this technique.

Fast atom bombardment (FAB) was the first attempt in this series. FAB MS can ionize relatively large polar molecules ranging to 10 kD. The plasma desorption mass spectrometry allows the characterization of protein up to 23 kD. The use of matrix-assisted laser desorption ionization (MALDI) and electron spray ionization (ESI) allows the characterization of peptides and proteins up to the size of 10^5 kD. Beimann et al. used FAB and collision-induced dissociation (CID) effectively to study the primary structure of thioredoxin and glutaredoxin. Mass spectroscopy in combination with NMR can give deep insight into the nature and population of transient folding intermediates of proteins.
Problems Associated with SPPS

The reactions in heterogeneous media are not like any true solution type reactions. Hence unless all the different stages in a multi-step synthetic procedure proceed unequivocally and quantitatively, we cannot expect pure product at the end of the synthesis. Even if we consider the chances of other side reactions such as racemisations during coupling, side reactions associated with individual amino acids, branching of peptide chain from the side chain of a trifunctional amino acid, and the problem of detachment of peptide from the support, the yield of peptide after 10 or 15 residue incorporation will be too low. The accumulation of significant amount of deletion peptides in a step-wise synthesis, and the difficulties in separating them from full length peptide are well-documented. The inherent problems which limit the yield of peptides have identified and these are attributed to the polymer effect or self aggregating tendency of the peptide bound to the support via $\beta$-sheet formation or a combination of both.

Polymer Effect

It was believed that polymer support does not have any influence on the attached peptide chain. However studies show that the polymer has a significant effect on the growing peptide. In SPPS methodology, the growing peptide chain is covalently attached to the insoluble support. The support imbibes organic solvents, and becomes highly swollen gel. Studies indicate that the interior of peptide-resin is highly a mobile environment comparable at
a molecular level to free solution, except the fact that diffusion is restricted, and inhomogenities within the swollen resin are averaged out in NMR time scale. The rate of incorporation of a particular amino acid has been found to decrease with the increasing peptide chain length. The slow reactivity has been attributed to the steric effect of polymer at various functional sites. Narita and co-workers studied the influence of peptide chain length in the coupling efficiencies of amino acid with terminal amino acid of the oligopeptide bound to soluble polystyrene, copoly(styrene-1% DVB), and copoly(styrene-2% DVB). They noted little influence of peptide chain length upto 10 residue amino acid length in the case of soluble and 1% crosslinked polystyrene. However coupling efficiencies was lowered with peptide chain length in the case of 2% crosslinked polystyrene. This may be due the decreased solvation of polymers in the higher crosslinked system. Free permeation of reactants and solvents into the polymer support is an essential condition for effective gel phase reaction. The decrease in coupling yield in SPPS is believed to result from the restricted permeation of carboxyl component into resin matrix. Another reason is that functional groups that are placed near the crosslinks experience steric effects and are inaccessible to the incoming reagent. Morawetz carried out extensive investigations on the reactivity of functional groups attached to polymer support. He pointed out that introduction of crosslinks in polymer support changes the local polarity of the system; however the degree of crosslinking did not exert any marked influence on reactivity. Ford et al. using NMR-pulse-gradient spin echo (PGSE) method showed that all reactive sites in polystyrene gels are not equally reactive. The SPPS kinetics reveal that the reactive sites for the first 90% reaction are effectively
homogeneous, but substantial reduction in rate was observed for the final 10%. Crosslinking of polymer cause the final stages of the reaction to be extremely slow.

Solvation studies on PS matrix reveal that polystyrene is hydrophobic and is compatible with hydrophobic solvents, but as the peptide chain length is increased the amide bond changes the polarity of the peptidyl resin. However Sarin et al. has demonstrated that polymer and peptide chains mutually enhance one another's solvation, and this is a supporting evidence for Flory's view.

Studies from this laboratory have shown that optimum hydrophobic-hydrophilic balance is required for the polymer support for effecting the solid phase reactions successfully. In this optimum conditions, the polymer will be compatible with the peptide chain even if the length of peptide is increased or the polarity of solvent is changed.

Bayer et al. introduced PEG as a support in order to avoid matrix effect of crosslinked system and the heterogeneous reaction. PEG owing to its good solubility in many organic solvents can solubilise even otherwise insoluble peptides. An advantage of PEG based liquid phase method is the possibility of carrying out a better analytical control of the reaction, since spectroscopic techniques like NMR, IR and CD can routinely be applied which will help to study the changes in conformation of peptide during synthesis. The problems involved in the process of separation of PEG-peptide from reaction media and insolubility problem in the case of larger peptides bound to PEG, hinder the wide spread use of this technique.
The introduction of PEG grafted crosslinked polystyrene (PEG-PS) was an attempt to overcome the above problems. There are two forms of PEG-PS. The anionic polymerization of ethylene oxide to attach the PEG chain of controlled length on crosslinked polystyrene bead containing OH group. This resin is marketed as tentagel resin.\textsuperscript{61,67} Preformed polyethyleneglycol has been attached to polystyrene bead to obtain PS-PEG. The PEG-PS in contrast to PS offer better solvation in protic solvents. The PEG, since not crosslinked, the reaction sites are highly accessible resulting in greater reactivity.\textsuperscript{61} The main limitations of PEG-PS in comparison to PS are higher cost, reduced loading level, and significant mechanical instability.

Atherton and Sheppard have investigated on the origin of the steric hindrance in polymer supported peptide synthesis.\textsuperscript{168} Based on the concepts of polymer-peptide structures they could explain the polymer effects on the reactivity in polymer-supported reactions. The four possible states of polymer (polystyrene) and peptide in solvents of varying polarity are shown in Figure 2.3.

Figure 2.3.(b) represents the condition when the reactions are carried out in a hydrophobic solvent. In this case the polystyrene matrix is extremely solvated, whereas the peptide chain, by virtue of its hydrophilicity due to the presence of the amide bond, is coiled up. The condition is reversed when hydrophilic solvents are used (Fig. 2.3.(c)). In this state most of the peptide chain though solvated effectively, are trapped in the shrunk polymer matrix. Figure 2.3.(d) is the condition where solvents of medium polarity are used. In
all these cases the end amino acid to which next amino acid is to be incorporated becomes inaccessible to the reaction, because of the steric reasons from either the polymer part or peptide part or from a combination of both. An ideal condition, where the peptide chain is highly available to the reagent is depicted in Figure 2.3(a). In this state the peptide and polymer are equally solvated. Based on this idea, Sheppard and co-workers developed a

Fig. 2.3. Possible states of polymer (PS) and peptide chain in solvents of varying polarity
(a) ideal condition (polymer and peptide chains are effectively solvated
(b) peptidyl resin in hydrophobic solvent (c) peptidyl resin in hydrophilic solvent (d) peptidyl resin in solvent of medium polarity

series of polyacrylamide based supports which are structurally similar to peptides.\textsuperscript{41-46} Though these supports are commercially available, they are very expensive. Another drawback is that high capacity resins are only seldom obtained.
Aggregation of Peptide

Besides the polymer effect described above, another important factor which is considered as a general source of problem in peptide synthesis is the insolubility (in classical peptide synthesis) or aggregation (in SPPS) of hydrophobic peptide due to freely interpenetrating coils. Internal aggregation of peptide chains bound to crosslinked polymer brings extra crosslinking to the system, reduces the swelling behaviour of polymer support, eventually leading to the inaccessibility of the reagents and solvents to the reactive groups in the matrix. The onset of aggregation in solid phase peptide synthesis can be characterized by:

- A series of reproducible incomplete amino acylations (0.5-15%).\textsuperscript{169}
- Recoupling or capping provides little or no improvement.\textsuperscript{162,169}
- Occurs irrespective of resin type or strategy.\textsuperscript{169-171}
- Difficulty increases with high resin loading.\textsuperscript{169,172}
- Aggravated by sterically hindered amino acid.\textsuperscript{169}
- Associated with specific sequences.\textsuperscript{169,172}

The internal aggregation has been shown to be antiparallel $\beta$-sheet structure by FT IR.\textsuperscript{173} Studies by Narita have shown that significant solubility of protected hydrophobic peptide was noted when Aib residues were incorporated into peptide chains.\textsuperscript{174-176} Although the use of Aib in peptide synthesis finds limited application, the above studies corroborate the idea that insolubility of protected hydrophobic peptide segment is due to $\beta$-sheet formation, since the $\alpha$-helix
promoting and β-sheet disrupting property of Aib is well-known. The view is supported by the Raman, FT IR and CD studies of protein aggregates.

There are three types of hydrogen bonding existing in peptidyl resin.

1. Peptide-polymer backbone interaction.

2. a. Intrachain interaction, which occurs frequently at reverse turns.
   b. Interchain interaction, formed when ordered structures such as helices, or β-sheet formed within peptidyl resins, creating a network of interchain hydrogen bonding.

3. Interchain interaction in the initial stage of synthesis between residues 5 and 15 where 3-4 consecutive residues encountered coupling difficulties which disappears after this junction.

Out of these, interchain interaction resulting in the formation of β-sheet structure is very severe as far as synthesis is concerned.

The mechanism of β-sheet structure in protein and peptide is less clear than that of α-helical structures, due to the lack of good isolated model system to test the theoretical prediction. Tam and Lu studied the coupling difficulty associated with interchain clustering using branched oligolysine peptide bound to polystyrene. Their studies reveal that the solvation of clustered model is very much reduced in polar and nonpolar solvents than a dispersed model. When the peptide content exceeds 50% of peptidyl resin there is transition from polystyrene to polyamide. Hence polar solvents offer better coupling yield than protic solvent.
Field et al. demonstrated that the solvation of peptide-resin is dependent on both growing peptide backbone and nature of side chain protecting group. Benzyl (BzI) based side chain protected amino acids are more prone to solubilisation in polar solvents compared to their alkyl based counter part. Therefore it can be inferred that Boc protecting scheme is superior to Fmoc protecting method. Because in Fmoc protection scheme the side chains are alkyl based, whereas in Boc strategy the side chains are protected by Benzyl groups. Also the FT-Raman spectroscopic investigation of peptidyl resin, shows that Na-protecting Fmoc group has influences on the secondary structure, supporting mainly $\beta$-sheet conformation, whereas Boc group has essentially no effect on the secondary structure of pendant peptide chains. However recent investigations on the influence of protecting groups on the $\beta$-sheet structure stability of protected peptides in organic solvents show that $<\text{SP}_\beta>$ value, which is a measure of the peptide chains’ propensity to form $\beta$-sheet structure, of protected peptides are almost independent of the kinds of protecting groups. Studies on the $\beta$-sheet nucleation by Kelly et al. have shown that the $\beta$-sheet formation is stabilized by amino acid with bulky hydrophobic residues like Val, Ile, and Leu and amino acid with aromatic side chain.

Narita et al. demonstrated that the stability of $\beta$-sheet structure in organic solvents is mainly under the influence of the nature of organic solvents and that of protected peptide. The electron donating and electron accepting abilities of solvents predominates the stability of the $\beta$-sheet structure. In the case of nature of protected peptide, the $\beta$-sheet stability is achieved by the nature of amino acid and the peptide chain length.

Based
on the solubilities of several hydrophobic peptides in different solvents, they have given an arbitrary value, \( SP_\beta \), - the \( \beta \)-sheet stabilizing potential value - for different amino acids, and based on this they have concluded that as the \( SP_\beta \) value increases the \( \beta \)-sheet structure becomes more stable. The \( \beta \)-sheet structure of protected peptide is strongly dependent on amino acid composition and weakly dependent on their sequence.\(^{192,193}\) Narita has proposed a method for the prediction of solubility of protected peptide and recommends this method in the design of synthetic routes.\(^{190,191,194}\) The close interrelation between conformation and physicochemical properties of a peptide was brought to light in the synthesis and conformational investigations of homo- and oligo-peptides.\(^{195}\)

It can be stated that aggregation could be limited in solid phase peptide synthesis, if low capacity resins were used, thereby minimizing interaction between peptide chains. Sarin et al. observed that the space availability for peptide chain growth within swollen resin beads is not a limiting factor in SPPS.\(^{196}\) But this will not be applicable, where intermolecular peptide interaction occurs. Narita has shown that intraresin site separation of peptide chain is not achieved even in swollen state.\(^{197}\) The \( \beta \)-sheet is formed even at low loading\(^{197}\) and the high dilution principle\(^{198}\) is not achieved in SPPS. Though hydrophobic polymers show a high degree of swelling in apolar solvents, where it favours \( \beta \)-sheet formation but polar supports (e.g. polyacrylamide) show high swelling in solvents which destabilizes or even disrupt \( \beta \)-sheet structure.\(^{199}\) However, the existence of \( \beta \)-sheet promoting site within the statistically functionalised resin appears to be an intrinsic problem.\(^{171}\) \( \beta \)-Sheet structures exhibit considerable stability under the conditions of peptide
synthesis\textsuperscript{153} and give rise to a variety of peptide products lacking one or more internal amino acids result in only low yield and cause purification problems\textsuperscript{169,200,201} and in most cases this leads to abrupt termination of the synthesis before the desired product is obtained.\textsuperscript{201} The state of peptide bound to polymer in $\beta$-sheet structure is shown in the following scheme (Scheme 2.1). It is obvious that though polymer tries to swell in the "good solvent" the peptide in the $\beta$-sheet suppress the process.

Scheme 2.1. $\beta$-sheet structure formation in SPPS

(a) peptidyl resin in solid state (b) solvation of polymer is suppressed by peptide in $\beta$-sheet structure (c) solvation of peptide & polymer, $\beta$-structure is minimized

With the development of Fmoc protecting groups, and the continuous flow method in automation the formation of $\beta$-sheet structure, and difficult sequence can be detected spectrophotometrically. IR, CD, NMR and GPC are also widely used for the detection of $\beta$-folded structure of peptide.\textsuperscript{202-205} Ludwick used solid state deuterium NMR method to study this
phenomenon.\textsuperscript{203} \textsuperscript{13}C NMR studies were carried out to investigate the conformation of peptides bound to soluble polymers.\textsuperscript{206}

Several methods have been proposed for the removal or disruption of $\beta$-sheet structure during peptide synthesis. Lithium bromide in anhydrous THF was found to be very useful for the destabilization of $\beta$-sheet structure.\textsuperscript{207,208} However, the rate of coupling is too low in this case. Other inorganic salts used are ammonium thiocyanate and perchlorate.\textsuperscript{209} These too are encountered with several problems and are not efficient in giving complete amino acid incorporation. Fluorinated alcohols—1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 2,2,2-trifluoroethanol (TFE)—have been found promising.\textsuperscript{201,210-212} But they are too expensive to be used widely.

There are reports that the use of NMP in combination with 15% DMSO gives the best result in coupling.\textsuperscript{190,213} Recently, Hyde et al. recommended the use of DMSO as a potential solvent for $\beta$-structure destabilisation.\textsuperscript{214} A drawback of this solvent is that DMSO is an oxidising agent and therefore should be used with caution when oxidisable amino acid like cysteine and methionine are used. However, earlier studies pointed out that the ability of DMSO to disrupt $\beta$-sheet structure of peptide is same as NMP, DMF and HMPA, while DCM containing small amount of TFE or HFIP has wonderful effect in removing the $\beta$-sheet structure compared to DMSO.\textsuperscript{215} Narita et al. suggested DCM containing TFE as a better reaction solvent than any other solvent.\textsuperscript{216} $\beta$-sheet structure could be disrupted even in DCM in presence of strong electron donor solvents like HMPA and DMSO.\textsuperscript{187,217} This solvent system has been recommended as coupling media in the case of highly
hydrophobic peptides.\textsuperscript{217} Narita have demonstrated that β-sheet structure disruption by organic solvents is strongly dependent on their electron-acceptor (AN) and electron-donor (DN) numbers and solvents having larger AN or DN values have high potential for β-sheet structure disruption.\textsuperscript{218} Recently Mutter showed that when PEG was used as the protecting group for amino acids the solubilising power of peptides bound to polymer was greatly enhanced.\textsuperscript{219} The incorporation of proline residue in a potentially secondary structure forming peptide disrupts the formation of β- as well as helical structures and increases their solubility.\textsuperscript{220,221} Based on this, the side chain of serine, threonine or cysteine were converted to oxazolidine/thiazolidine function, which mimic proline (pseudo-proline, ψ-Pro) and disrupts the β-formation when these amino acids residues are incorporated in a sequence.\textsuperscript{222-225} Along with the emergence of different tactics for the removal of aggregation, methods have been reported for the prediction of difficult sequences.\textsuperscript{216,217} With a forecast about the difficult stages that will be encountered during synthesis such methods help the peptide chemist in the design of the synthetic route more effectively and economically.

In the present work we have employed NMP along with DMSO (15%) as the coupling medium. This has been found to be very effective for all the synthetic procedures.