CHAPTER - 2

SOLID PHASE PEPTIDE SYNTHESIS –
A REVIEW ON RECENT DEVELOPMENTS
Polymer supported synthesis has achieved a major place in polypeptide and polynucleotide synthesis over the past three decades. Peptide synthesis has proven indispensable for the structural elucidation and activity studies of many naturally isolated products having a peptide structure such as hormones, neuropeptides, antibiotics and enzymes, which can be isolated only in very small quantities. Synthetic peptides find application in all areas of biomedical research including immunology, neurobiology, pharmacology, enzymology and molecular biology. The chemical synthesis of peptides with the naturally occurring structure is possible, it was used for the development of artificial vaccines and potent drugs that can substitute the conventional drugs having various side effects. Investigation of structure-activity relationship of biologically active peptides also demands the synthesis of many analogues of a given peptide.

In the beginning of 20th century, Emil Fischer synthesized the first peptide in solution. The general chemical requirements for the synthesis of peptide involve the blocking of carboxyl group of one amino acid and the amino group of the second amino acid. The activation of the free carboxyl group resulted the formation of amide bond between the amino acids and the selective removal of the protecting groups resulted a dipeptide. The method developed by Fischer was laborious and time-consuming because the intermediate peptides have to be removed, purified and characterized before the next coupling step. The major limitation of classical solution phase synthesis of peptides is the low yield and solubility of the intermediate peptides with increase in chain length. A new approach was needed for the synthesis of larger and more complex peptides with high purity and yield.

Merrifield introduced the concept of solid phase synthesis to achieve more efficient synthesis of peptides. In SPPS, the peptide chain was assembled in a stepwise manner while the C-terminal end of the peptide was anchored to an inert cross-linked polymer support and the peptide was grown from C-terminal to N-terminal residue. Merrifield demonstrated the feasibility of the idea by synthesizing a model tetra peptide L-leucyl-L-alanyl-glycyl-L-valine. Simultaneously with Merrifield, Letsinger and Kornet reported the synthesis of a dipeptide, L-leucyl-glycine on a "Popcorn polymer support"
using a different chemical strategy. The N-terminal amino acid was anchored to the polymer support and the peptide was grown from N-terminal to C-terminal. This technique is not so popular because the cleavage from the support under mild condition is not possible and always there is a problem of racemisation. Merrifield’s invention formed the basis of a new technique of peptide synthesis, which has been used till now with several methodological improvements and refinements. The design of polymer support, its chemistry and applications for SPPS have been extensively reviewed. These refinements and recent developments in designing solid supports and various factors involving in solid phase peptide synthesis are reviewed in this chapter.

2.1 Principles of Merrifield’s Peptide Synthesis

SPPS follows the strategy of the stepwise assembly of peptides by consecutive coupling of amino acids. The stepwise synthesis is carried out from the C-terminal to the N-terminal of the target peptide. This approach can eliminate the possibility of racemisation. The stepwise synthesis using low molecular C-terminal protecting group finds less application because of its sparing solubility in organic polar solvents does not permit a homogeneous reaction. Carrying out the synthesis as a heterogeneous reaction is also not very promising due to poor filterability of the reaction mixture.

Merrifield’s method employs an insoluble and filterable polymeric support such as cross-linked polystyrene that function as the carboxyl-protecting group for the C-terminal amino acid of the peptide. The target peptide sequence was formed in a stepwise manner by attaching temporary Nα-protected C-terminal amino acid to the chloromethylated PS-DVB resin. After the removal of Nα-protection, the next Nα-protected amino acid is coupled and the process is repeated until the entire desired peptide is assembled on the polymer support. Dicyclohexyl carbodiimide (DCC) is used as the coupling agent, and all the reactions are carried out under non-aqueous conditions in organic solvents. The target peptide was deprotected and cleaved from the polymer matrix by acidolysis with HF or anhydrous TFA in the presence of suitable scavengers.
Even though highly pure peptides can be synthesized by classical solution phase method, it has the following shortcomings:

i. The method is slow, tedious and laborious. In order to obtain a peptide with high purity, the constituent amino acids are incorporated in a stepwise manner starting from the peptide's C-terminus end. After each completed amino acid addition, the intermediate peptide is separated from any remaining reactants before its characterization, leading to lengthy synthesis time.

ii. The increasing insolubility of the growing peptide chain in the reaction medium causes problems in both purification and in the next coupling step results in the termination of peptide chain elongation.

iii. Methods such as chromatography and crystallization required during the synthesis results in considerable reduction of the overall yield of the peptide.

Solid phase peptide synthesis has the following advantages over the classical solution phase method.

i. The peptide is synthesized while its C-terminus is covalently attached to an insoluble polymeric support. This permits the easy separation of the growing peptide from any by-products or excess unused amino acid components.

ii. The reactions are driven to completion by using an excess of reactants and reagents.

iii. No mechanical loss occurs because the growing peptide is retained on the polymer in a single reaction vessel throughout the synthesis.

iv. The final peptide is detached from the polymer support by a single cleavage step at the end of synthesis. The side chain protecting groups can also be cleaved in the same reaction in order to simplify the work-up and the isolation of the final peptide. The cleavage step does not degrade the assembled peptide.

v. The physical operations involved in the synthesis are simple, rapid, and amenable to automation.

vi. The spent resin can be recycled.

In spite of these advantages, Merrifield's solid phase method has a number of limitations and is extensively reviewed.\textsuperscript{16,22-24} They are:

i. Non-compatibility of resin and growing peptide chain.

ii. Lack of stability of peptide-resin linkage under the conditions of synthesis.
iii. Non-equivalence of functional groups attached to the polymer support.
iv. Formation of error peptides due to truncated and failure sequences.
v. Peptide conformation changes in macroscopic environments inside the polymer matrix and also due to peptide resin linkage.

A number of modifications have been introduced to overcome the difficulties associated with Merrifield's SPPS which includes:

i. Development of new supports with high swelling properties permitting improved solvation of both matrix and growing peptide chain.
ii. Introduction of multi-detachable anchoring groups improving the flexibility of synthetic strategy.
iii. Development of newer separation method (eg. preparative and semi-preparative HPLC) and characterization techniques in peptide synthesis.

Extensive investigation upon reaction rates and kinetic course in solid phase synthesis revealed that the reaction sites within the polymeric matrix are chemically and kinetically not equivalent, resulting in deviations from linear kinetics. Consequently quantitative reactions are difficult to obtain. The preparation and accessibility of insoluble polymer reagents appears to limit a more general application of the compounds. These inherent difficulties in solid phase synthesis were mostly overcome by the "liquid-phase procedure" proposed by Bayer and Mutter, which makes use of soluble polymeric groups. These macromolecular groups determine the physical and chemical properties of low molecular weight components covalently attached to the polymer. Such a technique allows the effective and quantitative removal of reagents. All reactions proceed in homogeneous solution in analogy to the low molecular weight system. The reduced operational simplicity and changes in the crystallization tendency makes the liquid phase peptide synthesis less versatile.

2.2 The Role of Solid Support

The stability of the solid support under all conditions of functionalisation and synthesis is the prime requirement in SPPS. It is believed that the peptides showed a lower tendency to aggregate because of limited intermolecular interactions when bound
to a polymer than in real solution especially at low substitutional levels. SPPS could therefore be favoured for solution condensations when solubility problems in LPPS arise.\(^{31-33}\) The restricted mobility of the peptide anchored to the polymer could become a disadvantage of SPPS. The PS-DVB support has been widely used for the synthesis of peptides using Boc-chemistry.\(^{34-35}\) Polystyrene resin with 1% PS-DVB showed optimum swelling and stability while 0.5% PS-DVB resin is too fragile and above 2% PS-DVB resin does not swell effectively.\(^6\) The extent of swelling in polar organic solvents increases as the number of amino acid residues incorporated to the resin increases.\(^{36}\) This is due to the lowering of the network free energy based on the additional solvation of the growing peptide chain.\(^{33,37,38}\)

Merrifield’s technique has undergone a series of modifications and improvements because of the physicochemical incompatibility of the growing peptide chain and the rigid hydrophobic macromolecular environment created by the PS-DVB network of the support. In order to optimize the resin structure in SPPS, Sheppard introduced a polar polydimethyl acrylamide resin, which is structurally similar to peptide backbone.\(^{12,39}\) This helps easy solvation of the peptidyl resin and thus reduces the steric hindrance during deprotection and coupling reactions.\(^{40-43}\) Cross-linked and functionalised polydimethyl acrylamide gel can detained within the pores of fabricated Keiselguhr which can be used as a matrix in continuous flow method. The support shows effective swelling in polar solvents but in non-polar solvents it is very poor. The chemical stability of the resin was also less comparable to that of polystyrene supports.

The mixed PEG-PS resin, a highly promising class of solid support, is used successfully in polypeptide synthesis.\(^{44-46}\) A cross-linked polystyrene-polyethylene glycol graft co-polymer with a 2-nitrobenzyl anchoring group has been used as a solid support for the stepwise synthesis of peptides.\(^{47}\) Swelling, which is a sign of good solvation of the resin, is good for polystyrene resins in non-polar solvents like DCM, whereas polyacrylamide resins swell much better in DMF. Mixed PEG-PS polymers show excellent swelling in common solvents such as THF, acetonitrile and alcohols.\(^{44}\) The similar polarity of peptides and polyacrylamide support, both well solubilised in DMF, makes the support suitable for SPPS.\(^{48}\) On the basis of solvent-resin interactions,
increasing effort has been made to introduce polyacrylamide where initially polystyrene supports were used.

Bis-2-acrylamidoprop-1-yl polyethyleneglycol cross-linked dimethyl acrylamide (PEGA) has been introduced as a hydrophilic, biocompatible and flexible solid flow stable support in peptide synthesis (1).

The first flow stable synthesis resin was obtained by polymerization of the soft polydimethyl acrylamide gel inside a solid matrix of supporting Keisegluhr. Small and Sherrington replaced the irregular Keisegluhr with more regular rigid 50% cross-linked polystyrene sponge containing a grafted polydimethyl acrylamide gel. This technique was developed for grafting polyethylene glycol on to 1% cross-linked polystyrene, which are monodispersed, spherical and flow stable. Polystyrene grafted to films of polyethylene has been used for synthesis of peptides under non-polar conditions (2). Polyhydroxypropyl acrylate coated polypropylene and cotton has shown some promise as supports under polar conditions. A co-polymer of bis-acrylamido polyethylene glycol, N, N'-dimethyl acrylamide and acryloyl sarcosin ethyl ester was successfully employed for the synthesis of peptides. The inert polyethylene glycol cross-linked resins such as polyoxyethylene-polyoxypropylene (POEPOP) (3) and polyoxyethylene polystyrene (POEPS) (4) were efficiently used as flexible and biocompatible resins in
Different concepts for multiple peptide synthesis (MPS) or simultaneous multiple peptide synthesis (SMPS) were developed to respond the rapidly growing demands for a large number of peptides with completely different sequences. The various methods can be distinguished by the differences in the polymeric supports employed, the number of peptides possible and the amount of products obtained. The multiple synthesis methods are mostly applied in hormone and inhibitor research. For the identification of relevant side chains, every amino acid of a biologically active peptide can be systematically substituted, the chain length can be varied, and N-as well as C-termini can be modified.
In the initial work of SPPS, the various N-terminal modifications such as acetylations, biotinylations, succinimidilations or couplings for preparing immunogens could be achieved by multiple methods or by consecutive syntheses. In multiple peptide synthesis it is possible to simultaneously prepare the same peptide bound to different anchors and cleave to obtain peptide acid, peptide amide, alkylated amide, hydrazide or a fully protected fragment. The "tea-bag" method proposed by Houghten belongs to the oldest strategies of multiple peptide synthesis. In "tea bag" method, polystyrene in polypropylene mesh packets were used as supports. Geysen et al. developed the concept of multi-pin synthesis technology (PIN) and several hundred peptides can be simultaneously prepared using this procedure. Acrylic acid coated polyethylene rods were used as supports in multi-pin synthesis technology. Valerio et al. used 2-hydroxy ethyl methacrylate grafted polyethylene supports in multi-pin peptide synthesis. In multi-column methods Macrosorb-SPR resin was used. Frank et al. proposed an inexpensive procedure for the preparation of polymer bound peptides in which the first amino acid was coupled to a sheet of cellulose paper. Recently cross-linked enzyme crystals (CLECs) of thermolysin were also used for peptide synthesis.

The polymer matrix has a significant role in SPPS. The success of SPPS depends on the physicochemical characteristics of the peptide bearing support. For effective swelling of the resin and solvation of the peptide, the polymer should have an optimum hydrophobic-hydrophilic balance. The structure-reactivity and structure-property correlations in cross-linked polymeric systems helped to design new supports with mechanical stability and optimum reactivity. Pillai et al. developed a series of polymer supports by introducing polar cross-linking agents such as triethyleneglycol.
dimethacrylate (TEGDMA) tetraethyleneglycol diacrylate (TTEGDA, 5), and hexanediol diacrylate (HDODA) to polystyrene network. These resins have optimum hydrophobic-hydrophilic balance and swell like a gel in most of the organic solvents used in peptide synthesis. Efficiency of these supports was demonstrated by synthesizing large number of peptides in high purity and yield. The new member of this series described in this thesis, developed by cross-linking 1,4-butanediol dimethacrylate (BDODMA) with polystyrene. This support showed very high swelling characteristics in various solvents, an effective hydrophobic-hydrophilic balance and is successfully used for the synthesis of peptides. The optimum reactivity of these newly developed resins are due to the greater chain mobility of the cross-linker in solvents that enable effective interaction between the reactants and resin bound functional groups.

![Chemical structure](image_url)

(5)

2.3. New Approaches in Solid Phase Peptide Synthesis

2.3.a. Resin-peptide linkages

Polypeptide synthesis by solid phase technique is more effective when a specific combination of the linker/handle-resin was used. These linkers can help the cleavage of the peptide from the support under specific selected conditions that enable the peptide free from side reactions. The covalent linkage between the growing peptide chain and the polymer support is one of the factors that determine the purity of the peptide. The design
and development of a series of bifunctional linkers (handles) have facilitated and extended enormously the scope and application of SPPS approach for the preparation of large peptide through a convergent strategy\textsuperscript{71} and also hybrids of peptides with other biomolecules containing labile structures.\textsuperscript{72-74} One end of the bi-functional spacers is attached to a smoothly cleavable protecting group and the other end allows coupling to a previously functionalised support. The linkages are easily formed and stable to repeated cycles of acylation and deprotection steps.

The initial procedure of SPPS was based on temporary Boc and permanent Bzl-protection\textsuperscript{71} (Boc/Bzl) with benzyl ester linkage of the peptide to the resin. The HF cleavage of the peptide resulted in concomitant loss of all protecting groups, thus offering no access to protected peptide fragments. The protected peptides can be obtained from Merrifield resin by trans-esterification, ammonolysis or hydrogenation depending on the stability of side chain protection.\textsuperscript{75-81}

Recently Fmoc/\textsuperscript{Bu based protection schemes have become increasingly popular with the development of a great number of handles or linker-resins and cleavage of the protected peptide from the resin is achieved under milder conditions by a great variety of reagents. Wang developed p-alkoxy benzyl alcohol resin (6) for the synthesis of protected peptide fragments having a free carboxyl group and p-alkoxy benzyloxycarbonyl hydrazide resin (7) for the synthesis of protected peptide hydrazides.\textsuperscript{82,83} The p-alkoxy substituent of the benzyl alcohol moiety enhances the acid sensitivity between the peptide and resin and 50% TFA can cleave the anchoring bond. The supports (6 & 7) can be used for the peptide synthesis using Fmoc-amino acids in which $N_{\text{O}}$-protection was removed by an organic base.\textsuperscript{84,85}
4-hydroxyphenyl thiomethyl resin (8) can also be used for the SPPS by Boc-amino acids. The cleavage of the peptide from the resin can be achieved by using H$_2$O$_2$/acetic acid or by nucleophiles.

![4-Hydroxyphenyl Thiomethyl Resin](image)

(8)

Kaiser's oxime resin (p-nitrobenzophenone oxime ester resin) (9) had a major impact on SPPS. It allows the preparation of peptides following Boc-strategy. The final cleavage occurs with nucleophiles to yield hydrazides, N-alkyl amides, acids and peptide esters.

![Kaiser's Oxime Resin](image)

(9)

From Wang resins the N$_a$-protection, Boc was removed with 30% TFA in DCM, which results in a minute loss of peptide from the resin. This drawback causes difficulty in the synthesis of long peptides. More acid labile handles or linker-resins like (4-hydroxymethyl-3-methoxy phenoxy)acetic acid (10) and 2-methoxy-4-benzyloxy benzyl alcohol based resins (11, super acid sensitive resin, SASRIN) allow cleavage with 1% TFA/DCM, but can still cause loss of N$_a$ and side chain protection.

![4-Hydroxymethyl-3-Methoxy Phenoxy Acetic Acid](image)

(10)

![2-Methoxy-4-Benzylloxy Benzyl Alcohol Based Resin](image)

(11)
Barlos' trialkoxydiphenyl methyl ester resin (12), Rink resin (13), Barany's hypersensitive acid labile tris (alkoxy) benzyl ester anchoring (14,HAL) and Flersheimer's handle (15) are fully compatible with permanent Boc-protection of the peptide.\textsuperscript{96-100} There is a special acid-lability for handles 14 & 15 which allows unproblematic cleavage of the $N_a$-Boc and side chain protected peptides from the resin.

The resin containing oxymethyl phenyl acetamidomethyl handle (PAM-resin) (16) was used successfully in SPPS.\textsuperscript{101,102} The electron withdrawing linker makes the ester bond more acid stable than the simple alkoxy benzyl alcohol resin.
Protected peptide acids can be obtained by using the linkers [4-(2-hydroxyethyl)-3-nitro] benzoic acid (NBE) (17), hydroxycrotonoyl aminomethyl resin (18) and 3-(4-hydroxymethyl phenyl) 3-trimethyl silyl propionic acid (19). The cleaving agents used were a base for resin (17), Pd in basic nucleophiles such as N-methyl aniline for resin (18) and TBAF for resin (19).

The peptides esters can be effectively prepared by employing the 3-(4-hydroxy phenyl) propionic acid handle attached resin and 2-bromo acetyl phenyl resins. The peptides were obtained by trans-esterification in presence of a base.

Peptide amides can be prepared by the cleavage of benzhydryl amine-4-oxy carbonyl methyl resin (20) bound peptide with HF. Nucleophiles like aqueous or alcoholic hydroxide gives peptide acids, alcohols in presence of tertiary amine gives peptide ester, ammonia gives peptide amide and hydrazine gives peptide hydrazide.
4-hydroxymethylbenzamidomethyl resin (21), 4-hydroxymethyl phenoxyacetamidomethyl resin (22) and 3-methoxy-4-hydroxymethylphenoxy acetamidomethyl resin (23) are also used in SPPS.\textsuperscript{12,83,93,108} TFA treatment of the peptide bound resin under various conditions yield peptide acids.

Protected peptide acids can also be prepared from the support in which a base labile N-[(9-hydroxymethyl)-2-fluorenyl] succinamic acid (HMFS) handle (24) attached to a p-methyl benzhydryl amino (MBHA), aminomethyl polystyrene and aminomethyl polyethyleneglycol-polystyrene.\textsuperscript{109} Peptide can be cleaved from the resin by morpholine in DMF.
Muller and Barany developed N-(4)-[[[(4-hydroxymethyl)-phenoxy-t-butylphenyl)silyl]phenyl} pentanedioic acid monoamide (PBS) handle attached resin (25) for the synthesis of protected peptide acids. The peptide can be cleaved from the resin by tetrabutyl ammonium fluoride (TBAF).

The N^3-phenoxy carbonyl-L-2,3-diaminopropionic acid residue acts as a versatile linker (Dpr(Phoc) linker) (26) shows high stability under neutral and acidic conditions but undergoes activation under mild alkaline conditions for the release of peptide acids or amides by nucleophilic cleavage.

Protected peptide amides can be synthesized by the stepwise addition of Fmoc-amino acids to 4(2',4'-dimethoxy phenyl aminomethyl)phenoxy methyl resin (27). The cleavage of peptide can be affected by using acetic acid or dilute TFA.
Peptide amide can also be prepared by using 4,4'-methoxybenzhydryl phenoxy acetamide methyl resin (28), 5-(4-aminomethyl-3,5-dimethoxy phenoxy) valeric acid resin (29), 4,4',6-trimethoxy benzhydryl amine-3-propionic acid resin (30). TFA was used as the cleavage reagent of the peptide from the resin.

Acid labile 5-(9-Fmoc-aminoxanthene-2-oxy) valeric acid (XAL) resin (31) is also used for the synthesis of peptide amides.
2.3.b. Multi-detachable handle supports in SPPS

Multi-detachable handles were developed for facile cleavage of protected peptides from the resin allowing purification and reattachment of the peptide to the resin. These handles provide greater chemo-selectivity between the protecting groups of α-amino group and the anchoring bond, which binds the peptide to the resin support. The cleavage of the peptide at different positions from the multi-detachable resins can be achieved by the use of acids, nucleophiles or by photolysis. The peptide obtained as free acid, as a protected peptide, which is suitable for fragment condensation, or as a form which have a removable spacer suitable for the attachment to a resin support for further elongation.

Tam et al. have developed two resins 2-(4-hydroxymethyl) phenyl acetoxy propionyl resin (32) and 4-[4-(hydroxymethyl)phenyl acetoxymethyl]-3-nitro benzamidomethyl resin (33) which can be cleaved selectively by acidolysis at position (I) or by photolysis at position at (II).

\[ \text{(32)} \]

\[ \text{(33)} \]

Benzhydryl amine-4-oxycarbonyl methyl resin (34) is also considered as a multidetachable support in which an acid can cleave at position (I) and a variety of nucleophile at position (II). Thiophenoxide in DMF or aqueous/alcoholic hydroxide gives peptide acid, alcohol in presence of tertiary amine gives peptide ester, ammonia gives peptide amide and hydrazine gives peptide hydrazide.
2.3.c. Photolysis—an efficient method for the synthesis of protected peptides in SPPS

The rapidly growing field of combinatorial chemistry has renewed interest in the use of solid phase organic synthesis technique as a convenient means of assembling molecules. In addition to synthetic methods amenable to solid phase approach, the need for improved and novel linkers anchoring the molecules to the support has greatly expanded. Photolabile linkers have received considerable attention during the past 24 years. Peptides are complex molecules having several types of reactive side chains, chiral centers, acid and base sensitive amino acids and should be protected from possible side reactions during the final cleavage from the solid support. The use of acidic and basic reagents for cleavage leads to unwanted products. By employing a photolabile handle between the resins and growing peptide, the final peptide can be cleaved from the support as a fully protected form by photolysis at 350 nm in 20% TFE/DCM solvent mixer.

The photolytic cleavage becomes facile when the support contains a chromophore, which is sensitive to light and at the same time stable to the conditions of peptide synthesis. During the photolysis, the light should affect only the anchoring linkage between the photosensitive group and the peptide. The photolysis offers a mild method of cleavage that takes place under neutral conditions. This detachment is orthogonal to acidic and basic reaction conditions and therefore affords additional flexibility in the synthesis on solid support.

O-Nitrobenzyl compounds have been widely employed in the generation of peptides. The nitration of Merrifield resin at 0 °C with fuming nitric acid yield 2-nitrobenzyl resin. The protected amino acids are incorporated to the resin following...
the usual solid phase synthetic procedure. The finished peptide is cleaved from the support by photolysis (Scheme -2.1).

Scheme-2.1. Photolytic cleavage of peptide from the 2-nitrobenzyl resin.

Nitration enhances the polarity of the support whereas it reduces the swelling of resin in organic solvents with low dielectric constant. Introducing a handle containing photosensitive chromophore can eliminate this drawback. The photolabile 3-nitro-4-bromomethyl benzamidomethyl resin (35) and 3-nitro-4-aminomethyl benamidomethyl resin (36) can be successfully used for the synthesis of peptide acids and peptide amides respectively.

Protected peptide acids can be obtained by photolysis of peptide attached 4-hydroxymethyl-3-nitrobenzoic acid resin (Nb) (37) and 3-nitro-4-(1-bromoethyl) benzyl resin (38).
Phenacetyl and benzoin based linkers are also used as photolabile handles in SPPS. \( \alpha \)-methyl phenacetyl group has been used as a photosensitive anchoring group for carboxyl function.\(^{125} \) This handle-attached resin (\( 39 \)) acts as a photolabile support in SPPS.\(^{121} \) 3-N\(_\alpha\)Fmoc-amino-3-(2'-nitrophenyl)propionic acid-aminomethyl resin (ANP) (\( 40 \)) and 4-[4'\{1"-(Fmoc-amino)ethyl\}-2'-methoxy-5' nitrophenoxyl]butyric acid-aminomethyl resin (Fmoc-photolabile resin) (\( 41 \)) are used to generate amides and amines from the corresponding carboxylic acids or alkyl substrates.\(^{126,127} \)
4-(2-Bromopropionyl) phenoxy acetic acid resin (42) is also used as a photolabile support in solid phase strategy for peptide synthesis.

![Diagram of 4-(2-Bromopropionyl) phenoxy acetic acid resin (42)]

Recently, Peukert and Giese introduced a photolabile pivaloyl glycol-anchoring group (43) for solid phase peptide synthesis.

![Diagram of a photolabile pivaloyl glycol-anchoring group (43)]

2.3.d. Linkers for miscellaneous protection of peptides

With the rapid development of segment condensation technology and combinatorial chemistry, differently protected peptides like peptide aminoalkyl amides, peptide α-thioacids etc is required. The peptide alkyl amide Pseudoargiopinine III was synthesized in the solid phase strategy using an allyl linker. Canne et al. introduced thioester resin linker (44) for the solid phase synthesis of peptide α-thioacids.

![Diagram of thioester resin linker (44)]
For the solid phase synthesis of C-terminal modified peptides, a redox-sensitive resin linker was recently developed by Zheng et al. The redox sensitive linker attached resin (45) was proved to be successful in peptide synthesis. The cleavage of peptide from the resin can be achieved by treating with sodium hydrosulphite in water and THF for 3 h at room temperature.

2.3.e. Use of Fmoc-amino acids - a mild procedure for SPPS.

In conventional solid phase peptide synthesis individual t-butoxycarbonyl protected amino acids with benzyl ether, ester and urethane side chain protection groups are coupled sequentially to an insoluble gel-support. The temporary Nα protecting Boc-group can be cleaved by mild acid treatment and the anchor bond is cleaved by strong acid. The repetitive cleavage of Boc-groups by TFA or HCl-acetic acid may be deleterious in the synthesis of long peptides or proteins and also the nucleophilic side chain of Tyr, Trp, Met and His can undergo trifluoro acetylation during TFA treatment.

Sheppard et al. introduced a simple, rapid and exceptionally mild strategy in SPPS using base labile Nα-fluorenyl methoxy carbonyl amino acids with t-butyl based side chain protecting groups and p-alkoxy benzyl ester resin linkages. This method can overcome some of the problems experienced in Merrifield's method. Reactions using conventional solid supports are influenced by dissimilar solvation properties of the polymer and growing peptide chain causes the decreased rate of peptide bond formation and deprotection. In order to eliminating these drawbacks Sheppard and co-workers developed polyamide supports that are structurally similar to peptide backbone. These supports show high swelling behaviour in polar aprotic solvents such as DMF, NMP etc. Fmoc-groups can easily be cleaved by non-hydrolytic base like piperidine and the final cleavage of peptide from the resin can be achieved by using neat TFA. Fmoc-group has been shown to be completely stable to acids like TFA, HBr/acetic acid or HBr/nitromethane.
2.3.f. Polymeric carboxyl protection

The choice of attachment to the support through the carboxyl group of the C-terminal amino acid reflects the prevailing philosophy of peptide chemistry. The C-terminal amino acid was attached to the polymeric support through an ester bond. In classical Merrifield's synthesis, the esterification reaction was carried out by heating TEA salt of Boc-amino acid in ethanol with chloromethyl resin. The slow reaction rate, incomplete esterification reaction and the formation of quaternary ammonium group on the polymer are the main limitations of the method. The use of more sterically hindered base such as ethyl diisopropyl amine minimises the quaternary salt formation. The esterification reaction can be driven to completion, if tetraethyl ammonium salt or cesium salt of Boc amino acids are used.

Another side reaction of chloromethyl group involves alkylation of histidine, methionine and cysteine during esterification reaction. If ethanol was used as solvent for esterification reaction, some ester interchange may occur with aspartic acid and glutamic acid. To avoid the difficulties of direct esterification, hydroxymethyl resins or resins with suitable linkers containing a hydroxyl functional group were used. Various methods were adopted for the attachment of Boc/Fmoc amino acids to the resins. Esterification with symmetric anhydride of Boc/Fmoc amino acid was the most commonly used method and 4-dimethyl amino pyridine was used as the catalyst. Less than one equivalent of catalyst was required, it can able to promote hydrolysis of activated Fmoc amino acids, and reaction with resin bound hydroxyl group simultaneously. DMAP also significantly promotes racemisation of activated urethane protected amino acids and also cleave Fmoc-protecting group leading to the formation of dipeptide. These drawbacks can be eliminated by repeating the esterification reaction with fresh reagents after 50 min if the esterification was not completed within 50 min. Advantages of Fmoc-SPPS include milder reagents, ability to monitor Fmoc-deprotection by UV method, and in general, higher yields of desired peptide. However, it is considerably more expensive than Boc-SPPS. Miller and Scalant introduced a method for SPPS based on a new α-amino protecting group, o-nitrobenzene sulfonyl group (oNBS) that retains compatibility with Fmoc-SPPS and offers several additional advantages. The oNBS offers a number of
exemplary features that support its use as a temporary amino protecting group. The advantages are:

a) Deprotection of oNBS-protected peptides releases a yellow chromophore, which allows simple visual confirmation of deprotection.

b) oNBS-SPPS allows operational and selective N-methylation of the oNBS-protected nitrogen during peptide synthesis.

c) oNBS amino acid chlorides can be used to couple with extremely hindered amines on a solid support where the analogue Fmoc-amino acid chlorides fail to couple well.

d) The reagent necessary to synthesize oNBS amino acids, 2-nitrobenzene sulphonyl chloride is commercially available and considerably cheaper than Fmoc chlorides.

2.3.g. **Sequential attachment of amino acids and deprotection**

The factors depends on the efficiency of coupling reactions include the nature of acylating agent, protected amino acids activated species and the solvation of the resin-bound growing peptide chain. Subsequent amino acids must be added to the growing peptide-resin in a highly activated form to ensure their rapid and quantitative coupling. The most common activating species used in solid phase synthesis are the amino acid symmetrical anhydride and the amino acid active ester with HOBt. The symmetrical anhydride method using DCC (1) provides the derivatives of greatest reactivity, but their instability and the formation of insoluble DCU during acylation are the important drawbacks. Diisopropyl carbodiimide (DIPCDI, 2) and t-butyl ethyl carbodiimide (3) are the other carbodiimdes used in SPPS.¹⁴⁰,¹⁴¹

\[
\text{N=CH=CH}
\]

(1)

\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{N=CH=CH}_2 \\
\text{H}_3\text{C} & \quad \text{N=CH=CH}_2
\end{align*}
\]

(2)
The symmetrical anhydride method suffers a number of disadvantages such as:

a) They must be formed immediately before use because of their instability.
b) Amino acids are wasted during the anhydride formation because 2 moles of amino acids are required for each mole of symmetrical anhydride produced.
c) The most efficient solvent (DCM) for rapid symmetric anhydride formation is not the solvent of choice (DMF/NMP) for peptide synthesis.
d) The possibility of racemisation.

EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) (4) have also been used for the coupling of successive amino acids in SPPS.\textsuperscript{142}

The limitations of symmetrical anhydride method can be overcome by using the pentafluorophenyl (Pfp) (5), HOBt (6) and 3-hydroxy-2,3-dihydro-4-oxo-benzotriazine (oDhBt) (7) esters of amino acids.\textsuperscript{143,144} These esters suppress the racemisation during coupling reaction.
Benzotriazol-1-yl-oxy-tris(dimethyl amino) phosphonium hexafluoro phosphate (8) along with DLEA is one of the most advancing acylating agents in peptide synthesis.

The synthesis of hydrophobic peptides are difficult because of internal aggregation of constituent amino acids via β-sheet formation or by association between protected chain with the synthetic support matrix. The use of 2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyl uroniumhexafluorophosphate (HBTU) (9) or 2-(1H- benzotriazol-1-yl) 1,1,3,3-tetramethyl uroniumtetrafluoroborate (TBTU) (10) during the coupling reaction can avoid these problems. The coupling reaction proceeds rapidly with low level of racemisation. HOBr can act as a catalyst for this coupling reaction and the base like DIEA can activate the reaction.

1-Hydroxy-7-azabenzotriazol (HOAt) (11) and its uronium and phosphonium salt derivative such as o-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uroniumhexa-
fluorophosphate (HATU) (12), o-(7-azabenzotriazol-1-yl)-1,1,3,3-bis (tetramethylene) uroniumhexafluorophosphate (HAPyU) (13), 7-azabenzotriazol-1-yl oxytris-dimethyl amino-phosphonium hexafluorophosphate (AOP) (14), 7-azabenzotriazol-1-yl oxytris-pyrrolidino-phosphonium hexafluorophosphate (PyAOP) (15) etc in presence of DIEA can be used as acylating agents in acylation reactions.\textsuperscript{149}

The reduction in product loss, minimisation of failure sequences, greater control of coupling steps, increased reaction efficiency, decreased racemisation, reduced coupling times and especially suited to the preparation of the peptides containing hindered amino acids are the advantages of HOAt and its derivatives.
2.3.h. Detachment of peptide from the polymer support

Boc-SPPS is designed primarily for simultaneous cleavage of the peptide anchoring linkage and side chain protecting groups with strong acid, while Fmoc-SPPS is designed primarily to accomplish the same cleavages with moderate strength acids. In both cases scavengers are used in order to minimise the side reactions. Time required for the cleavage of peptide from the resin depends on the anchoring group between the peptide chain and the solid support. Peptide can be cleaved from the resin in fully protected form or with unique peptide carboxyl termination by using photolysis, fluoride ion, alkali or hydrogenation. Strong acids such as HF and TFMSA simultaneously cleaves PAM and MBHA linkages and removes the side chain protecting groups commonly applied in SPPS.\textsuperscript{150,151} Yajima and co-workers reported that an Fmoc synthesized peptide-resin can be cleaved and deprotected with trimethyl silyl bromide (TMSBr)/TFA in presence of thioanisole, m-cresol and ethanedithiol (EDT).\textsuperscript{152} A mixture of trimethyl silyl trifluoro methane sulphonate (TMSOTf), TFA and thioanisole can also be widely used as a cleaving agent.\textsuperscript{153} Neat ethylamine (EtNH\textsubscript{2}) cleaves Boc-derived peptides from benzyl ester linkages of a resin, which results in peptides with a C-terminal ethyl amide and protected side chains. The benzyl ester type linkers can also be cleaved by 2-dimethyl amino ethanol or N,N-diethyl hydroxylamine forming side chain protected peptide acids.
References


