Introduction to Fmoc Solid Phase Peptide Synthesis

I. Resins

Solid phase peptide synthesis (SPPS) is conducted mostly on functionalized spherical resin beads made up of polystyrene crosslinked with 1% divinylbenzene. Attached to each functional site of the polystyrene core is a linker (see **Figure 1**). The density of linkers attached to the resin is the resin's **loading** (or **substitution**), usually given in mmol/g (number of linkers in millimoles per gram of resin, where 1 mole = 6.02 x 10^{23}). Typical loadings fall between 0.2 – 1.0 mmol/g, with the most common loadings close to 0.5 mmol/g. Linkers "link" the peptide to the resin bead during solid phase synthesis (**Figure 2**).

Figure 1: Linkers (in black) attached to functional sites of resin bead.

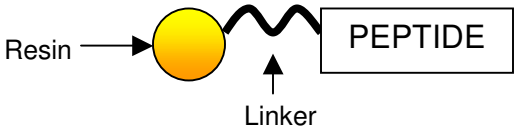

Figure 2: Close up of a single linker (black wavy line) linking resin to peptide.

There are different types of linkers which determine the properties of the final product and what chemicals the resin-bound peptide complex can be exposed to during reactions and cleavage. The most widely used linkers in Fmoc-synthesis are Wang and Rink. Wang resin consists of a polystyrene core with Wang linkers attached and produces a peptide acid (COOH). Attaching the first amino acid to Wang resin is different from the subsequent amino acid to amino acid attachments. It can be more time consuming and difficult, so to save the user time, **preloaded** Wang resins with the first amino acid already attached are available**.** Rink resin consists of a polystyrene core with Rink linkers attached and produces a peptide amide (CONH2). The first amino acid attachment is no different from subsequent amino acid to amino acid attachments. Solid phase peptide synthesis can be performed at the 0.005 – 0.250 mmol scale on the Symphony, and at the 0.005 – 0.350 mmol scale on the Prelude. To calculate the grams of resin required for a given scale, divide the scale (in mmol) by the resin loading (in mmol/g).

II. Solvents & Swelling

The most common solvents for Fmoc SPPS are dimethylformamide (DMF) or N-methylpyrrolidone (NMP). Prior to beginning a synthesis, the resin should be swelled in the synthesis solvent. Resins are like a sponge. Swelling allows them to expand and maximize the exposure of their functional groups to the incoming reactants. Resins are typically exposed to the synthesis solvent from 10 to 30 minutes for swelling.

III. Amino Acids & Protecting Groups

Amino acids have an amino group on one end, and a carboxylic acid group on the other end, hence the name amino acid. These groups can **couple** or attach to one another (think lego blocks, see **Figure 3**). The end of the peptide with the amino group is known as the **N-terminus** (amino terminus), and the end of the peptide with the carboxylic acid group is known as the **C-terminus** (carboxylic acid terminus). Peptide sequences are typically written in the N to C direction. However, peptides are attached to resin from their C-terminus, and are therefore synthesized in the C to N direction. In order to attach one amino acid at a time, the amino group must be protected with an **Fmoc protecting group** (**Figure 4**). Otherwise, the amino acids would form chains by linking to one another and the resin indefinitely. However, protecting the amino group is not enough. Amino acids also have unique side chains, some of which contain reactive groups. These reactive groups must be protected with various **side chain protecting groups** in order to prevent them from reacting and creating unwanted side products (**Figure 4**)

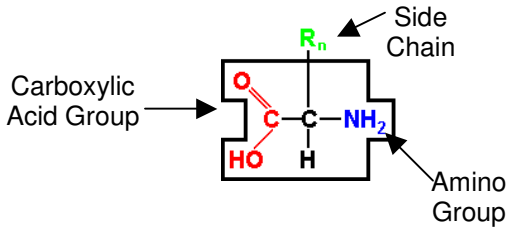

Figure 3: Amino acid. The carboxylic acid group is shown in red, the amino group is shown in blue, and the side chain is shown in green.

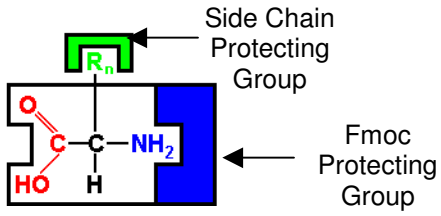

Figure 4: Amino acid with protecting groups. The side chain protecting group is shown in green, while the Fmoc protecting group is shown in blue.

IV. Deprotection

Deprotection is the first step in a synthesis cycle (**Figure 5**). It removes the Fmoc protecting group from the resin-linker (in the case of Rink resin) or attached amino acids (in the case of Wang resins, or an existing peptide chain). Fmoc groups are removed by treatment with base for 2 to 20 minutes. Typically, 20% piperidine in DMF is used. For difficult deprotections, a stronger base, 2% DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene) in DMF may be used.

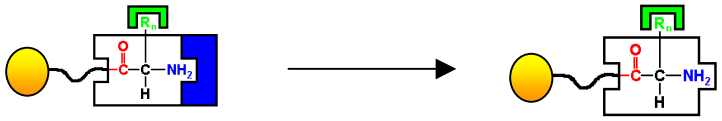

Figure 5: Deprotection step: Removal of the Fmoc protecting group from the N-terminus of the resin-bound amino acid (or peptide).

V. Coupling

V.1 Activation

In order for the carboxylic acid of an amino acid to attach or **couple** to the amino group of another amino acid, it must first be activated. In order to speed up the coupling reactions, an excess of reactants is added. This can range anywhere from 2 to 10 equivalents of amino acid per equivalent of resin. Typically, in small-scale syntheses, an excess of 5-10 (5-10 equivalents of amino acid per equivalent of resin) is used. For example, for a synthesis at the 25 µmol scale, 125 µmol of amino acid would be added during a coupling at 5 times excess. This could be accomplished by adding 1.25 mL of a 0.1 M amino acid solution to the resin [(100 mM/L (amino acid concentration)* 0.00125 L (volume)* 1000 µM/mM)/5 excess = 25 µM (scale)]. The amino acid solution would require activation, however. Usually, the amino acid/activator ratio should be 1:1 to ensure the amino acid is fully activated. Various activators include HBTU, HCTU, PyBOP, HATU, HOBt/DIC and DCC[1]. A basic solution is necessary for the first four activators to work. In general, the activator/base ratio should be 1:2 to 1:4. Bases used during coupling are NMM (N-methylmorpholine) or DIPEA (diisopropylethylamine). Typical amino acid solution concentrations range from 0.1 to 0.5 M in DMF. Amino acid solutions may be stored for up to a week at room temperature before they break down, but may be kept longer if refrigerated. Fmoc-L-Cys(Trt)-OH solutions should be used immediately, as they can break down within days. Protein Technologies, Inc. recommends activating a 0.1 M solution of amino acid dissolved in DMF with an equal volume of coupling solution: 0.1 M HBTU/0.4 M NMM in DMF.

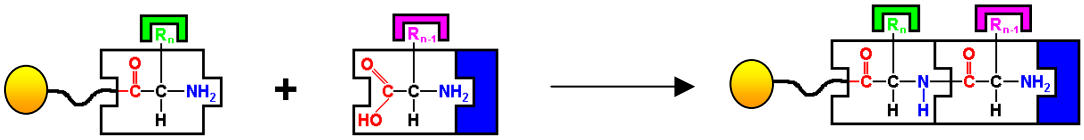

Figure 6: Coupling step: Activated protected amino acid is coupled to the resin-bound amino acid (or peptide).

V.2 Coupling Efficiency

Coupling efficiency determines the final purity of the peptide. >99.5% coupling efficiency is necessary to obtain sufficiently pure crude peptide, especially in long peptides. Lower coupling efficiencies result in low yields and peptides with low purity. Coupling reaction times can range from 5 minutes to 2 hours depending on the activator used, the amino acid being coupled, and the sequence of the peptide. The reason the peptide sequence has an effect is because certain sequences tend to aggregate, or clump together, hiding reactive sites on the end of the peptide chains. Various methods help reduce the effects of aggregation including using different solvents such as DMSO (dimethylsulfoxide) or NMP, or chaotropic salts. Using resins with greater solvation capacity helps too, as

[1] **HBTU** = 2-(1H-Benzotriazol-1-yl-)-1,1,3,3-tetramethyluronium hexafluorophosphate, **HCTU** = 1H-Benzotriazolium 1-[bis(dimethylamino)methylene]-5chloro-hexafluorophosphate (1-),3-oxide, **PyBOP** = Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, **HATU** = 2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphate, **HOBt** = 1-hydroxybenzotriazole, **DIC** = N,N'-Diisopropylcarbodiimide, **DCC** = Dicyclohexylcarbodiimide

does using resin with low substitutions (around 0.2 mmol/g). Aggregation typically occurs when the amino acid side chains are hydrophobic, or as the peptide chain gets longer.

VI. Washing

It is important to wash the resin between deprotection and coupling steps. Otherwise, residual piperidine could remove the Fmoc group from the amino acid being introduced. Protein Technologies, Inc. recommends washing the resin with the primary solvent (DMF) 2 to 6 times for 30 seconds.

VII. Capping

Capping is performed to permanently block any unreacted amino groups following a coupling reaction, or to acetylate the N-terminus of a completed peptide. It is useful during the synthesis of difficult or long peptides to minimize deletion products. Typical capping solution compositions include 1:1:3 acetic anhydride/base/DMF, where pyridine or DIPEA may be used as the base.

VIII. Final Deprotection & Cleavage

After synthesis is complete, the final Fmoc protecting group and side chain protecting groups need to be removed, and the peptide needs to be removed, or **cleaved** from the resin. The final deprotection is performed with 20% piperidine in DMF to remove the final Fmoc group while the peptide is still attached to the resin. Cleavage of the peptide from the resin and removal of the side chain protecting groups is accomplished with trifluoroacetic acid (TFA). It is common to add additional reagents to the TFA which are used as **scavengers** of the side chain protecting groups to prevent their reattachment to the peptide. Typical scavengers include EDT (ethanedithiol), water, thioanisole, TIS (triisopropylsilane), and phenol. Different scavengers are necessary for different side chain protecting groups and are therefore chosen based on the peptide sequence. The following table illustrates suggested cleavage "cocktails" (mixtures of TFA and scavengers).[2]

	Cocktail	Time
Peptides containing all amino acids except Arg(Mtr), Cys(Trt), Met, and unprotected Trp	TFA/TIS/water 95:2.5:2.5 (v/v)	1.5-3 h
Peptides containing all amino acids except Arg(Mtr) and/or unprotected Trp	TFA/TIS/water/EDT 94:1:2.5:2.5 (v/v)	1.5-3 h
All peptides	TFA/thioanisole/water/ phenol/EDT 82.5:5:5:5:2.5 (v/v)	1.5-18 h

[2] Reproduced from W.C. Chan and P.D. White, Ed. Fmoc Solid Phase Peptide Synthesis: A Practical Approach, Oxford University Press, Oxford, 2000, p. 66.

Cleavage reactions typically take 0.5 to 3 hours depending on the linkers and protecting groups used. After the cleavage reaction, the cleavage solution is filtered from the resin and the peptide is obtained by precipitation with ice-cold ether. If a minimal amount of TFA cocktail was used during the cleavage (10-20 mL per gram of resin), ether can be added directly to the TFA cocktail. If the peptide does not precipitate, there may be an excess of TFA. Evaporate off the excess TFA and ether and try again. Typically, 10 volumes of ether are added per one volume of TFA. Peptides should be washed at least 3 times with ice-cold ether following precipitation to remove the scavengers as much as possible. Afterwards, peptides may be left in a hood to air-dry overnight, or lyophilized for storage.

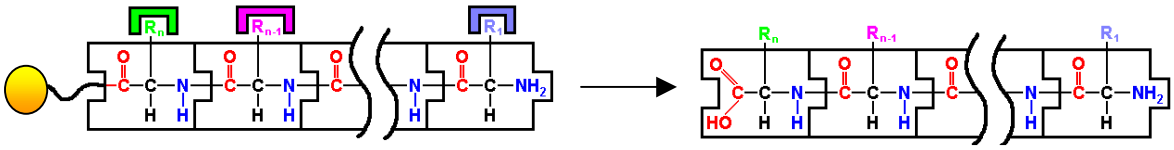

Figure 7: Cleavage step: Following the final deprotection, side chain protecting groups are removed, and the peptide is liberated from the resin.

IX. Analysis

IX.1 Yield

Percent yield can be calculated by dividing the dry mass of the peptide by the theoretical yield of the peptide. To calculate the theoretical yield of the peptide (in mg), multiply the scale (in mmol) by the molecular weight of the peptide product (in mg/mmol).

IX.2 HPLC

Percent purity can be determined using reverse-phase HPLC analysis. Peptides are dissolved at a concentration of 1-3 mg/mL in water or buffer A. A typical buffer system used for analysis of peptides consists of Buffer A = 0.1% TFA in water, and Buffer B = 0.1% TFA in acetonitrile. Generally, peptide samples are analyzed and purified by increasing the gradient of buffer B from 5-95% over 60 minutes on a column (C4, C8 or C18). UV Detection is at 214-220 nm. % purity is calculated by dividing the area of the product peak by the total area and multiplying by 100%.

IX.3 Mass Spectrometry

The molecular weight of the product can be verified by mass spectrometry. Various methods exist including MALDI-TOF, as well as ESI. The MALDI-TOF methodology requires samples to be spotted on a plate with a matrix solution. α-cyano-4-hydroxycinnamic acid is the most common peptide matrix.

X. Suggested Additional Reading

1. W.C. Chan and P.D. White, Ed. Fmoc Solid Phase Peptide Synthesis: A Practical Approach, Oxford University Press, Oxford, 2000.

2. E. Atherton and R. C. Sheppard. Solid Phase Peptide Synthesis: A Practical Approach, Oxford University Press, Oxford, 1989.

3. G. B. Fields and R. L. Noble. (1990) *Int. J. Peptide Protein Res.* **35**, 161-214.

4. M. Bodanszky. Peptide Chemistry: A Practical Textbook, Springer-Verlag, New York, 1988.