CHAPTER I

INTRODUCTION AND OBJECTIVES

The prospect of total chemical synthesis of peptides and proteins has always been a powerful tool in bio-organic chemistry. Recent interest in this area has been prompted by the use of peptides as antigens and synthetic vaccines and synthetic proteins and peptide segments as probe materials to decipher the structure-activity correlations. Further, peptide synthesis has become an indispensable tool in immunology studies. The solid phase approach of peptide synthesis developed by R. B. Merrifield\(^1\) in 1963 has helped to overcome many of the preparative difficulties encountered in the conventional method of peptide synthesis. This novel and widely used approach continues to be the method of choice for the synthesis of biologically active peptides and protein sequences. The chemical synthesis of peptides containing the twenty coded amino acids by the well-established solid phase technique, has made tremendous progress during the last two decades through refinements of the original strategy. Novel supports, new protecting and anchoring groups, coupling methods and reagents, cleavage and deprotection reagents, monitoring techniques, automation and better purification and analytical facilities offered by the currently available sophisticated instrumentation have
made the solid phase peptide synthesis an emerging area of intense research activity.

Although peptide synthesis on insoluble solid supports gained popularity in recent years, some of the problems are yet to be tackled and remedies recommended. One of the ever-challenging problems involved in the practice of peptide synthesis is the solubility problem attached to the peptide-bearing support during the synthetic cycle. The divinylbenzene-crosslinked polystyrene support originally employed by Merrifield, being hydrophobic in character, gets solvated only in non-polar solvents. As the stepwise incorporation of amino acids on the polymer supports progresses, solubility characteristics change dramatically due to the presence of the polar growing peptide chains attached to it. Sheppard and co-workers suggested that the incompatibility of the support with the growing chain could be circumvented by the introduction of polar, polyacrylamide-based supports. These supports with structures parallel to those of polar, aprotic solvents like dimethylformamide and dimethylacetamide were employed by the Sheppard group for the synthesis of a number of biologically active peptide sequences. However, these supports are mechanically fragile and can swell only in polar media. The problems of incomplete coupling and lack of reactivity at various stages of the multistep synthesis which led to the formation of error peptides continued to hamper peptide chemists.
Even if the side reactions are avoided, complete peptide coupling and amino deprotection at every stage of the multistep synthesis is highly unlikely due to the heterogeneous character of the macromolecular matrix. Drastic drop in reaction rates and yields can result from the macromolecular matrix effects and the dynamic changes in conformation\textsuperscript{6,7}. In a reaction involving a heterogeneous macromolecular system, functional group accessibility to the low molecular substrate may not be uniform at all reaction sites due to their non-equivalent distribution in the polymer matrix. Functional groups situated close to the crosslinks are not readily accessible to reagents in the continuous phase resulting in sluggish reaction rates. These observations have necessitated the design and development of novel supports with amphiphilic characteristics, compatible with the solvent systems usually employed in peptide synthesis. The polymer support does not remain inert or passive during the wide variety of synthetic manipulations. It interacts with the surrounding medium. In addition to holding the reactive function, it plays a key role in dictating the reactivity of the functional moieties\textsuperscript{8,9}. Systematic investigations have shown that the reactivity of the polymer-appended functional groups is a function of its fine molecular character. This, in turn, depends on a number of variables of polymer synthesis such as the nature of monomers, nature and degree of crosslinking, hydrophilic/hydrophobic balance of supports, swelling and solvation behaviour, porosity and pore
dimensions as well as the morphological characteristics. An in-depth study of the interdependence of these parameters on the macromolecular reactivity would help the development of 'tailor-made' polymeric supports ideal for peptide synthesis.

OBJECTIVES OF THE PRESENT WORK

After giving an overview of the state-of-the-art of solid phase peptide synthesis, the thesis describes the following physicochemical and synthetic studies:

1. Correlation of the macromolecular structural parameters with the reactivity of the functional groups relevant for peptide synthesis.

The effect of the following structural parameters on the functional group reactivity was investigated.

a. Nature of the monomers and resulting polymeric system.
b. Nature of the crosslinking agent.
c. Degree of crosslinking.
d. Spacer-effect between the matrix and the reactive function.

By a judicious choice of the monomers and crosslinking agent and introduction of the crosslinker in appropriate mole ratio by suitable polymerisation techniques, the hydrophilic/hydrophobic balance of the support, its swelling and solvation behaviour as well as the topographical
features can be adjusted. When the hydrophilic polyacrylamide chains are crosslinked with rigid and hydrophobic crosslinkers like divinylbenzene, the hydrophilicity of polyacrylamide is reduced considerably. Similarly hydrophobic polystyrene when crosslinked with the flexible, hydrophilic triethyleneglycol dimethacrylate (TEGDMA), the hydrophobicity of the polystyrene backbone is diminished considerably. In other words, the extent of hydrophilic/hydrophobic crosslinker in the copolymer precisely dictates the balance between hydrophilicity and hydrophobicity merged together in the polymer network. Moreover, the nature and extent of crosslinking decides the degree of swelling and solvation and also the specific morphology.

2. Probing the optimum conditions for effective synthetic conversions.

The utility of a polymer as a support for peptide synthesis largely depends on the efficiency of each synthetic step involved, which is dependent on the reactivity of the functional moieties. Quantitative conversion in every step could be possible if the functional moieties are all equally reactive. In order to delineate the structural requirements which would dictate the optimum reactivity of the functional groups towards the peptide bond formation step, investigations based on model reaction involving aminolysis of polymer appended amino functions by low molecular active esters were carried out. Polymeric
amines undergo aminolysis by p-nitrophenyl active esters forming a peptide bond and liberating 4-nitrophenol which could be monitored continuously by spectrophotometry. This reaction resembles the peptide bond formation by the active ester method.

3. Delineation of the structural parameters for efficient supports and illustration of the application of suitable resins for peptide synthesis.

The reactivity of the polymer-bound functional groups towards a specific reaction depends largely upon the microenvironment of the functions in the macromolecular matrix. They should be mobile and freely accessible and should in no way be engaged in any form of physical binding. The specific structural environments can sometimes influence the pendant functional groups to undergo loose physical interactions such as H-bonding. Synthesis of a few model peptides and biologically relevant sequences were carried out on suitable supports. The following polymeric supports were prepared in order to investigate the effect of the structural parameters on the efficiency of the peptide synthesis:

a. N, N'-methylene bisacrylamide (NNMBA)-crosslinked poly(acrylamide)s.

b. Tetraethyleneglycol diacrylate (TTEGDA)-crosslinked poly(acrylamide)s.

c. Triethyleneglycol dimethacrylate (TEGDMA)-crosslinked poly(acrylamide)s.

d. Divinylbenzene (DVB)-crosslinked poly-(acrylamide)s
e. NNMBE-crosslinked poly(acrylamide-dimethylacrylamide) terpolymer.

f. DVB-crosslinked poly(acrylamide-dimethylacrylamide) terpolymer.

g. TEGDMA-crosslinked poly(styrene)s

These resin supports after suitable functionalisation by polymer-analogous reactions were tested for their suitability in the synthesis of model and biologically relevant peptides.

**SUMMARY OF RESULTS**

**i. Synthesis of acrylamide-based polymeric supports**

Polyacrylamide (PA) crosslinked with varying mole proportions of NNMBE, TTEGDA, TEGDMA and DVB were prepared by radical-induced solution polymerisation. They were amino functionalised with ethylenediamine and hexamethylenediamine.

The amino capacities of the copolymers were determined titrimetrically. A definite dependence of amino capacity on the nature and extent of crosslinking was observed. DVB-crosslinked resin showed decreased capacities due to the network rigidity imposed by DVB crosslinks. Generally as crosslink density increased, amino capacity decreased. Highly hydrophilic TTEGDA and TEGDMA induced flexibility to the network and crosslinked copolymers containing these...
crosslinks gave high values of amino capacity. The widely different polarities and hydrophilicities arising out of the molecular compositions of the support affect their swelling and solvation characteristics also. The morphological variations of these systems were studied with the help of scanning electron microscopy. The vast variations in their surface topography arise out of the discrete changes in the molecular character of the supports.

By the introduction of a second commonomer - dimethylacrylamide - into the polymerisation recipe, terpolymers of NN MBA- and DVB-crosslinked poly(acrylamide-dimethylacrylamide)s were prepared. These polymers were aminofunctionalised and the capacity determined. These studies were carried out in order to find out whether separation of the pendant carboxamide groups in acrylamide copolymers by introducing N,N'-dimethyl carboxamide groups would increase the capacity and reactivity of the resulting terpolymer. The neighbouring carboxamide groups in DVB-crosslinked poly(acrylamide)s, due to the rigidity of the system, may assume conformations which favour the formation of H-bonds between the carbonyl oxygen and the amide nitrogen. However the reactivity was not improved.

11. Polymer reactivity

The reactivity patterns of the functional copolymers towards peptide bond formation was followed by the
aminolysis of polymeric amino functions with an active ester-N-benzoyl glycine 4-nitrophenyl ester. A comparison of the coupling efficiency of the various amino functionalised crosslinked poly(acrylamides) and the dependence of crosslink density on their reactivity was undertaken.

Maximum coupling was observed for the DVB-crosslinked poly(acrylamide)s. This is probably due to the attainment of an effective balance between the hydrophilic acrylamide backbone and the hydrophobic DVB crosslinks. The molecular character of 20% DVB-crosslinked acrylamide is such that a near-quantitative coupling efficiency was observed. These observations indicate the strong dependence of polymer reactivity on fine molecular character of the network. The nature of solvent and the duration for aminolysis also play a prominent role in deciding the extent of reaction. A 1:1 mixture of dioxane and water was found to be most suitable in terms of polarity.

The effect of introducing a spacer to separate the reactive function from the matrix was investigated. The functionalised aminohexyl resins showed decreased capacities and reactivities. This could possibly originate from the rigidity of the matrix induced by the introduction of additional crosslinks formed by double transamidation at either ends of the long hexamethylenediamine.
iii. Water-binding studies and hydrophilicity scale of the polymer supports

The acrylamide-based polymer systems are highly compatible with water. The interaction with water and aqueous-organic solvent mixtures is important in the synthetic reaction leading to the formation of peptides and also in the biological activity of the peptides. Therefore a systematic study of the water-binding of the resins was undertaken. The polymer supports synthesised above can imbibe large volumes of water within their pores forming highly swollen hydrogel networks. These hydrogels can retain the sorbed water at room temperature. The water sorption is a time/temperature-dependent process and takes 6-7 days for equilibration. The equilibrium water contents (EWC) of these swollen hydrogels were determined.

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\text{EWC} = \frac{\text{weight of water imbibed by gel}}{\text{total weight of hydrated gel}} \times 100
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The EWC values reflect the dependence of the water-sorption behaviour of the support on the nature of monomers as well as nature and degree of crosslinking. The phenomenon of water structuring and the resulting phase changes in these swollen hydrogels was studied as a function of their molecular character with the help of differential scanning calorimetry. The relative amounts of the different states of water in the swollen gels, namely the
loosely bound "freezing water" and the more tightly bound "non-freezing water" were estimated from the melting endotherms.

The quantity and states of bound water in the hydrogel determine the mechanical properties and transport phenomena in the gel\textsuperscript{11}. These studies were attempted to understand the water imbibition based on molecular structure and to evolve a hydrophilicity scale for the polymer supports under investigation. These studies are relevant in the application of swollen hydrogels for gel-phase peptide synthesis, a situation which mimics the biosynthesis of proteins within the ribosomal factories of RNA in the biosystems. The molecular character of polymers play a decisive role in their water-structuring behaviour.

iv. \textit{Polystyrene-based supports}

Hydrophobic polystyrene was crosslinked with the hydrophilic crosslinker TEGDMA\textsuperscript{12} in varying mole proportions by suspension polymerisation\textsuperscript{13}. They were functionalised by chloromethylation and the chlorine capacity determined. The molecular character and the degree of crosslinking dictate the extent of chloromethylation. The swelling and solvation behaviour of these supports was studied in a variety of polar and apolar solvents. These resins due to their specific molecular design and architecture are amphiphilic in character. They swell well in both the polar
and the apolar solvents usually employed in peptide synthesis. The presence of the rigid polystyrene backbone renders these supports mechanically stable withstanding all the repetitive operations involved in the synthetic protocol without physical attrition. The stability of the resin crosslinks under the peptide synthetic conditions was established by comparing the IR absorption spectra of the characteristic groups of the resins before and after treatment with trifluoroacetic acid (TFA). All characteristic absorptions remain unaltered showing that the diester crosslinks are stable under the conditions of peptide synthesis.

v. Peptide synthesis

The new TEGDMA-crosslinked polystyrene supports were successfully employed for the synthesis of model and bioactive peptides following the conventional Boc benzyl ester strategy of Merrifield\textsuperscript{14}. The first amino acid salt was esterified to the chloromethyl resin to form an acid-labile benzyl ester linkage. Subsequent amino acid units were assembled in a stepwise manner to form the desired sequence. The free target peptide was cleaved from the support with the help of neat TFA. The following peptides were synthesised on the poly(acrylamide) and polystyrene-based supports:
a) H-Gly-Ala-OH  
b) H-Ala-Ala-Gly-Gly-OH  
c) H-Phe-Leu-Leu-OH  
d) H-Phe-Leu-Pro-Leu-Ile-Leu-Arg-Lys-Ile-Val-Thr-Ala-Leu-OH (Crabrolin – a peptide toxin)  
e) H-Ala-Leu-Ala-Leu-OH (Drug targeting peptide)  
f) H-Lys-Val-Leu-Gly-OH ((12-15) segment of hGRF)  
g) H-Thr-Pro-Arg-Lys-OH (Contraceptive peptide)  
h) H-Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala-OH (Delicious peptide)  
j) H-Pro-Lys-Leu-Leu-Lys-Thr-Phe-Leu-Ser-Lys-Trp-Ile-Gly-OH ((28-40) segment of Seminalplasmin)  

Syntheses were carried out on 1, 2 and 4% crosslinked polystyrene supports. Only Boc-protected amino acids were used except in the case of side chain protected ones. Couplings were carried out in dichloromethane using dicyclohexylcarbodiimide activation. 1-Hydroxybenzotriazole was used wherever necessary. When 1% TEGDMA-crosslinked polystyrene was used, couplings were almost complete in the first step. However, a second coupling ensured the completion of reaction. All couplings were monitored by the semiquantitative ninhydrin test. Purity of the peptide was tested by TLC. Crude peptides were purified by Fast Protein Liquid Chromatography using reverse phase columns. Amino acid analysis was done after hydrolysing the
samples with 6N HCl for 22 h. Sequencing of the purified peptide was performed on an automated gas phase protein sequencer. Syntheses of seminalplasmin segments (14-26) and (28-40) were achieved on lightly crosslinked polystyrenes. Synthesis of the (28-40) segment of seminalplasmin was accomplished in high yield and purity compared with a similar synthesis on 1% Merrifield resin.

From systematic studies of the effects of variation in the nature of monomers, nature and degree of crosslinking, hydrophilic/hydrophobic character of the monomers on the reactivity of the polymer-appended functionalities, it can be reasonably concluded that suitable changes in the structural parameters of the macromolecular matrix would result in the design of ideal supports for peptide synthesis. The reactivities of the functional groups under different microenvironments were largely influenced by adjusting the crosslink density, hydrophilic/hydrophobic balance, porosity, rigidity and mechanical integrity. Peptide synthetic efficiency is dependent on these macromolecular structural parameters.

ORGANISATION OF THE THESIS

After a brief introduction of the solid phase method of peptide synthesis and its limitations, the objectives of the present work are stated in the opening chapter. Chapter two gives a critical account of solid phase peptide
synthesis and the present-day trends of the strategy with emphasis on the role of the structural parameters of the macromolecular matrix in efficient synthesis of peptides. The third chapter describes the synthetic and physicochemical studies of the various macromolecular systems selected for the present investigations. The syntheses of model and bioactive peptides are discussed in the fourth chapter. The experimental section described in chapter five consists of two Parts; Part A describes the experimental methods employed for the syntheses and physicochemical studies of the macromolecular supports; the experimental details of the syntheses of model and bioactive peptides are given in Part B. Chapter six summarises the important findings of the present investigations. References to the cited literature are listed at the end.
SOLID PHASE PEPTIDE SYNTHESIS: AN OVERVIEW