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Stepwise Polypeptide Synthesis of Neuropeptide GLU-PRO-PRO-GLY-GLY-SER-LYS-VAL-ILE-LEU-PHE ON PS-NVP-HDODA RESIN

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Abstract
A new terpolymer was developed by the radical aqueous suspension polymerisation of 1,6-hexanediol diacrylate, N-vinylpyrrolidone (NVP) and styrene(PS-NVP-HDODA). The resin had a very good hydrophobic-hydrophilic balance and showed high mechanical stability, and swelling properties. The utility of the new polymer was confirmed by the stepwise synthesis of ***neuropeptide Glu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe***

I. INTRODUCTION

Merrifield's 1963 contribution to science served to foment, decades later, an explosion in the field of polymer supported organic synthesis. He envisaged that covalent anchoring of the peptide chain to an insoluble and inert polymer should greatly improve the quality and quantity of the target peptide. Merrifield introduced a lightly crosslinked polystyrene- divinyl benzene polymer having a pendant chloromethyl group as the point of attachment, which has come to be known simply as the Merrifield resin. But its rigid nature, inadequate solvation in polar organic solvents, non linear kinetic behaviour due to the non uniform distribution, non accessibility of the functional sites buried within the hydrophobic core of the polymer to substrates and other problems associated with heterogeneous reaction condition leads to the formation of truncated and deletion sequences.[2] Justifiably or not, the physical property of swelling is considered to be of utmost importance in dealing with solid support. A new terpolymer was developed by the radical aqueous suspension polymerisation of 1,6-hexanediol diacrylate, N-vinylpyrrolidone (NVP) and styrene(PS-NVP-HDODA). The resin had a very good hydrophobic-hydrophilic balance and showed high mechanical stability, and swelling properties. The utility of the new polymer for the stepwise synthesis of acyl carrier protein.

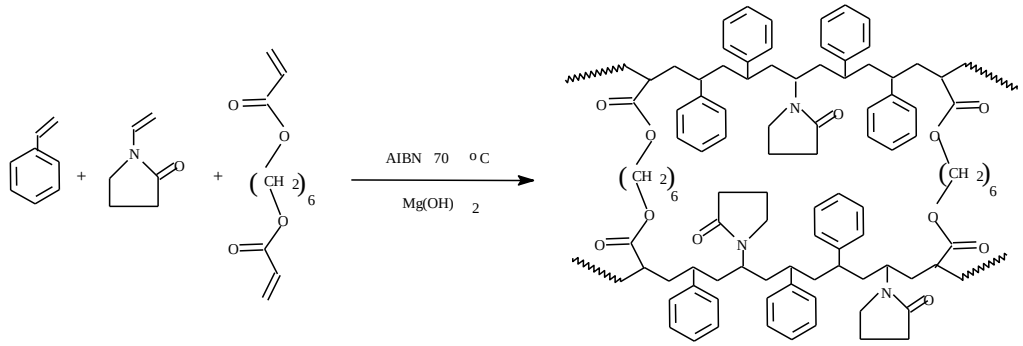
II. MATERIALS AND METHODS

Styrene, 4-(Dimethylamino) pyridine (DMAP), cesium carbonate, Sheppard resins (Novasyn® KA 125),dicyclohexylcarbodiimide (DCC), 2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU),Boc and Fmoc-amino acids, HMPA, HOBt, and MSNT were purchased from Novabiochem Ltd., UK. Thioanisole, 1, 6-hexanediol diacrylate (HDODA), ethanedithiol, diisopropylethylamine (DIEA), TFA, 4-(hydroxymethyl) 3-(methoxy) phenoxy butyric acid (HMPB), piperidine were purchased from Sigma-Aldrich Corp., USA. N-Vinylpyrrolidone and 1,6-Hexanediol diacrylate were purchased from E.Merck, Germany.Chloromethylmethyl ether (CMME) was prepared using literature procedure.[21] Solvents (HPLC grade) used were purchased from E. Merck (India) and BDH (India). IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The ^{13}C NMR measurements were conducted on a Bruker 300 MSL instrument operating at 75.47MHz. HPLC was done on a Pharmacia instrument using C-18 reverse phase semi- preparative HPLC column. Amino acid analysis was carried out on an LKB 4151 Alpha plus amino acid analyser. For this, the peptide was hydrolysed using 6N HCl in a pyrex glass tube fused under N_2 for 15 hours at 130^{o}C.

The Polymer Synthesis

The crosslinked polymer was synthesised by free radical aqueous suspension copolymerisation of the monomers styrene, N-vinylpyrrolidone and 1,6-hexanediol diacrylate. The amount of these monomers was selected according to the mole ratios required to make a definite percentage polymer. Magnesium hydroxide and sodium sulphate were added to the suspension medium (Scheme 4.2). Mechanical stirring was provided to form small uniform droplets of the dispersed monomer mixture suspended in the non solvent phase. The polymerisation reaction was initiated by adding radical initiator AIBN. It got solubilised in the monomer droplets and promoted the thermally induced polymerisation reaction. The temperature of the medium was raised to 70^{o}C to initiate the polymerisation process and the medium was kept at this temperature till the

polymerisation was completed. The bead size distribution of the polymer was found to be affected by the stirring rate, geometry of the reaction vessel and amount of the stabiliser.



Scheme Synthesis of PS-NVP-HDODA polymer

Chloromethyl PS-NVP-HDODA support

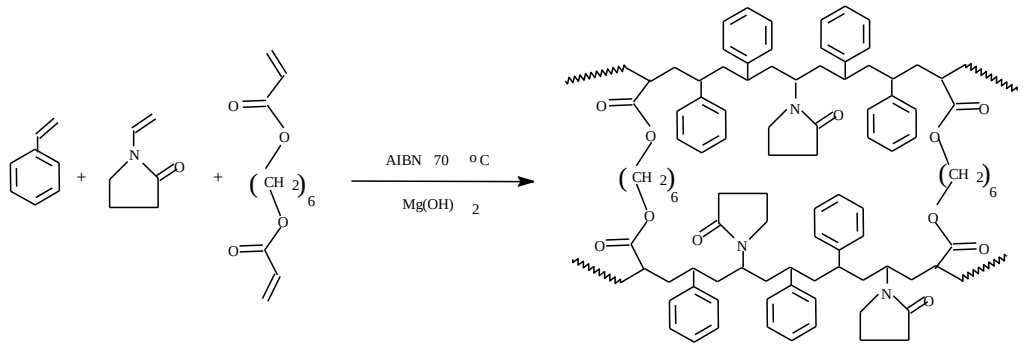
PS-NVP-HDODA support (4g) was swollen in DCM (50 ml). After 1 hour excess DCM was filtered off. The swollen resin was shaken with CMME (24 ml) and 1M $ZnCl_2$ in THF (0.6 ml) for 2 hours at 50°C. The resin was filtered using a sintered glass funnel, washed with THF (4 x 30 ml), THF/water (1:1) (3 x 30 ml), THF (3 x 30 ml), methanol (3 x 30 ml) and then soxhletted with THF and methanol.

Estimation of halogen content in functionalised PS-NVP-HDODA resin (Volhard's method)

Chloromethyl PS-NVP-HDODA support (100 g) was digested with pyridine (3 ml) in a Kjeldahl digestion flask for 3 hours at 100-110°C. It was quantitatively transferred to a 125 ml conical flask using 50% acetic acid (30 ml). Con. HCl was added followed by slow addition of standard $AgNO_3$(0.1N) solution (10 ml) with magnetic stirring. Water (50 ml) was added to the mixture. The excess $AgNO_3$was determined by back titration with standard ammonium thiocyanate solution (0.1N) using ferric alum as indicator till a dark brown colour was obtained. A calibration titration was carried out with standard NaCl solution. From the titre values the halogen capacity of the resin was calculated. Capacity of the resin = 0.24 mmolCl/g as estimated by Volhard's method.[25] IR (KBr): 1724, 1686 cm^{-1} (ester) and 1256 cm^{-1} (CH_2-Cl).

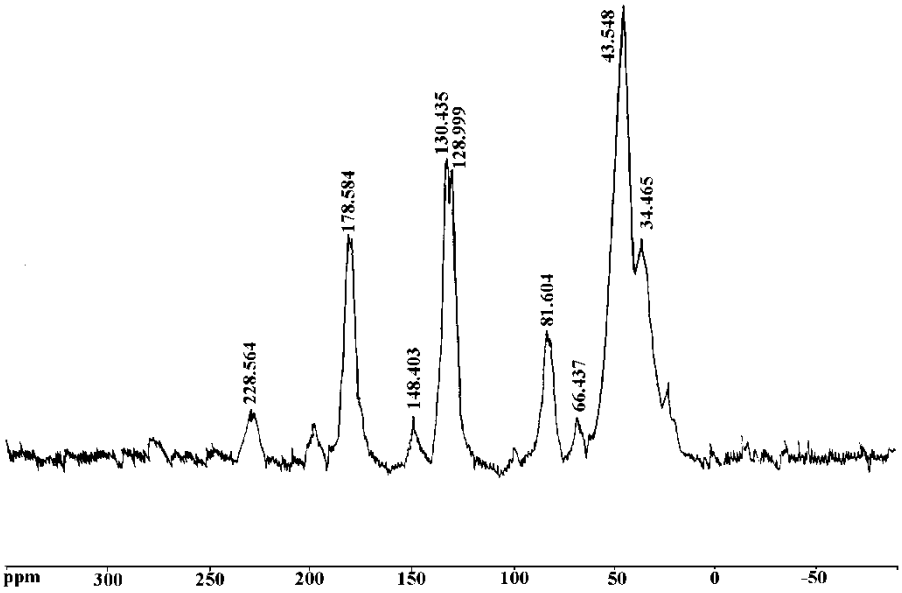
III. RESULTS AND DISCUSSION

The crosslinked polymer was synthesised by free radical aqueous suspension copolymerisation of the monomers styrene, N-vinylpyrrolidone and 1,6-hexanediol diacrylate. The amount of these monomers was selected according to the mole ratios required to make a definite percentage polymer. Magnesium hydroxide and sodium sulphate were added to the suspension medium (Scheme 4.2). Mechanical stirring was provided to form small uniform droplets of the dispersed monomer mixture suspended in the non solvent phase. The polymerisation reaction was initiated by adding radical initiator AIBN. It got solubilised in the monomer droplets and promoted the thermally induced polymerisation reaction. The temperature of the medium was raised to 70°C to initiate the polymerisation process and the medium was kept at this temperature till the polymerisation was completed. The bead size distribution of the polymer was found to be affected by the stirring rate, geometry of the reaction vessel and amount of the stabiliser.

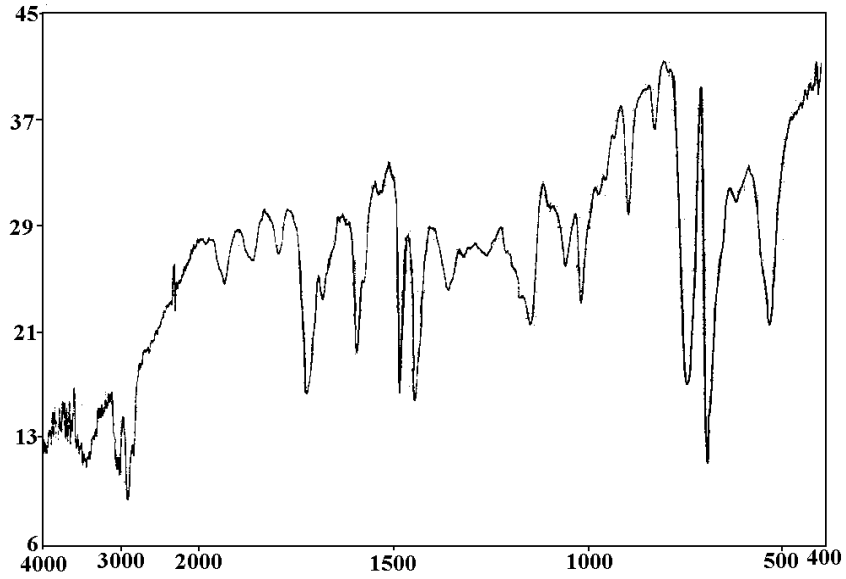


Synthesis of PS-NVP-HDODA polymer

PS-NVP-HDODA polymer was characterised by IR and ^{13}C/ NMR techniques. IR (KBr) spectrum of the powdered polymer showed an intense sharp peak at 1724cm^{-1} corresponding to ester carbonyl of the crosslinker and at 1686 cm^{-1} corresponding to the carbonyl peak of NVP besides the usual peaks of polystyrene. The solid state ^{13}C NMR spectrum showed an intense peak at 130.435ppm corresponding to aromatic polystyrene carbons and a small peak at 148.403 ppm corresponding to C-3 of styrene. The carbonyl carbon of the PVP appears as a peak at 178.584 ppm, methylene carbon of the crosslinker appears as a peak at 66.437 ppm. The peak at 43.548 ppm corresponds to the backbone methylene carbon of the polymer and that at 34.465 ppm was due to the overlapping of the ring with main chain carbon.



^{13}C/ NMR of PS-NVP-HDODA polymer support



IR (KBr) spectrum of PS-NVP-HDODA polymer support

In the initial phase of the development of the new resin, chloromethylfunctionalisation was used and it was carried out using CMME (Scheme 4.3). CMME was prepared as described in the literature procedure.Chloromethyl group was introduced to aromatic ring of the resin by a Friedel-Craft type electrophilic substitution reaction using $ZnCl_2$ as the Lewis acid catalyst. Anhydrous $SnCl_2$ showed very high catalytic activity. The reaction with anhydrous $SnCl_2$ was very fast, and so the controlled functionalisation of the resin using this catalyst was very difficult. Chloromethylation with $SnCl_2$ produced a change in the colour of the resin. This colour change of the polymer can cause many problems in peptide synthesis, especially during the ninhydrin colour sensitive reaction that helps to monitor the extent of coupling reactions. Chloromethylation was found to proceed smoothly when anhydrous $ZnCl_2$/THF was used as the catalyst. The degree of

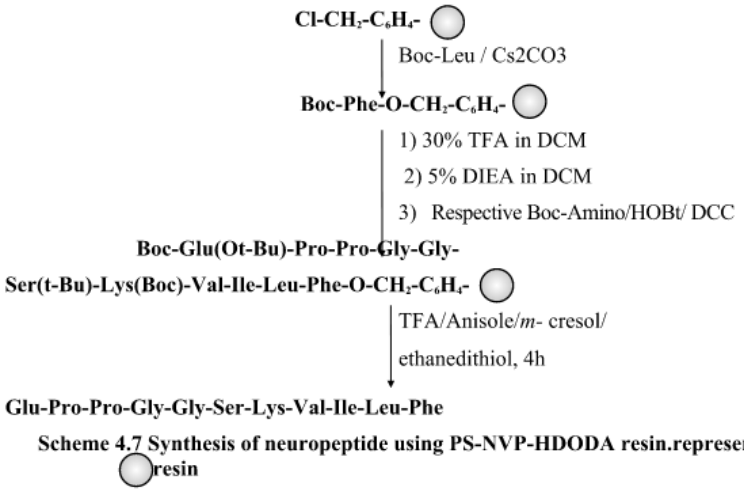
functionalisation was estimated by Volhardt's method.When the chloromethylation of resins having higher NVP percentage was carried out with anhydrous $ZnCl_2$/THFcatalyst, there was a chance for the formation of a Zn-NVP complex. This formation resulted in a change of colour to the polymer. Washing of the resin with various solvents could not remove this colour, that confirmed the high stability of this complex.

In Boc- strategy the C-terminal amino acid can be incorporated to the chloromethyl resin either by triethylamine method or by cesium salt method.[26]Intriethylamine method one molar Boc-amino acid was mixed with 0.9 molar triethylamine and the mixture was refluxed with chloromethyl resin for 24 hours using ethyl acetate as solvent. This method may result in the quarternisation of the C-terminal amino acid with resin bound functional group and the resulting resin bound quaternary salt later can act as an ion exchange resin. This can create problems during the peptide chain elongation process.

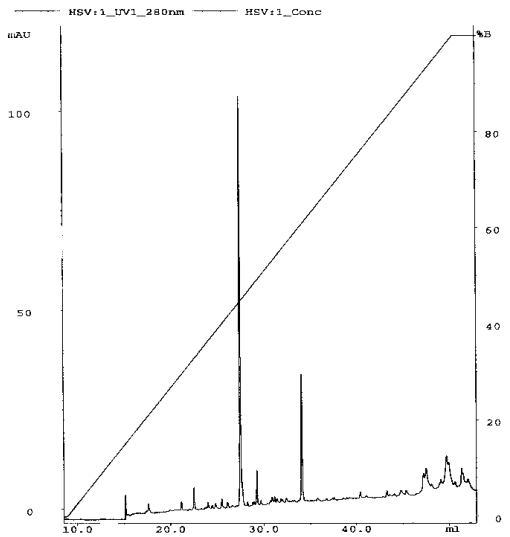
In the second technique, Boc- amino acid was incorporated to the resin as its cesium salt. The cesium salt of the Boc-amino acid was heated with the resin at 60°C for 24 hours with occasional stirring. After deprotection of Boc group, amino capacity was determined by picric acid method.

Synthesis of neuropeptide Glu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe

Boc-Phe was attached to chloromethyl PS-NVP-HDODA support by cesium salt method. After removing Boc protection, successive amino acids were incorporated as the HOBt active esters of the respective amino acids (Scheme 4.7). A second coupling was performed to ensure completion of the coupling reaction. Each coupling reaction was monitored by semi-quantitative ninhydrin test. After the synthesis, the peptide was cleaved from the support by treating with cocktail of TFA, phenol, water, thioanisole and ethanedithiol. The crude peptide obtained was dissolved in water, deep freezedand lyophilised. HPLC profile showed a major peak corresponding to the target peptide and a minor peak corresponding to a deletion.



Scheme 4.7 Synthesis of neuropeptide using PS-NVP-HDODA resin.represents the resin



HPLC profile of 11 residue neuropeptide. Solvent A: 0.5% TFA in water, B: 0.5% TFA in acetonitrile, Gradient used:0% -100% 40 min; Flow rate: 1 ml/min.

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