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Chemical Synthesis of Modularly Modified Analogs of E2/NS1 Peptides on a Novel Chemically and Mechanically Stable Terpolymer of 1,6-Hexanediol Diacrylate, N-Vinylpyrrolidone on Styrene Support

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Abstract

Analysis of the chemokine analogs synthesised on a terpolymer-polystyrene, N-vinylpyrrolidone, 1,6-hexanediol diacrylate crosslinked resin (PS-NVP-HDDA) showed it can be used a good solid support for the chemical synthesis of polypeptides. The polymer with a good swelling character and chemical resistance was synthesised by the radical aqueous suspension polymerisation technique. HPLC analysis of the crude peptides synthesised on PS-NVP-HDDA resin showed that it is a better substitute for most of the currently available polymer support for peptide synthesis.

Keywords: Polymer, Resin, Polystyrene, N-vinylpyrrolidone, Peptide

Introduction

Selection of polymer support is considered as a vital element in determining the homogeneity as well as the purity of biomolecules synthesized on it, including polypeptides and oligonucleotides. In 1963 Merrifield made a scientific discovery that helped spark a boom in polymer-supported organic synthesis decades later [1]. Later various attempts have been made for developing new polymeric supports with increased polarity and swelling compared to the Merrifield resin [2-5]. This article comprises the application of a newly synthesised terpolymer- polystyrene, N-vinylpyrrolidone, 1,6-hexanediol diacrylate resin (PS-NVP-HDDA) in the synthesis of 19-residue modularly modified fragment of E2/NS1 region of Hepatitis C viral polyprotein.

In most cases, serological HCV detection was performed using an ultra-sensitive PCR (Polymerase Chain Reaction) [6]. But the study required extra care and also quite expensive. Owing to these 2 drawbacks, another sensitive method was developed for detecting the anti-viral anti-bodies present in the sera from a patient with a "synthetic peptide ELISA developed using 19-residue fragments of the E2/NS1 region of Hepatitis C viral polyprotein [7]. The present study is spotlighted on the synthesis of modularly modified analogs of E2/NS1 region on the newly developed terpolymer polystyrene, N-vinylpyrrolidone, 1,6-hexanediol diacrylate resin (PS-NVP-HDDA) developed by radical aqueous suspension polymerization of styrene. The presence of 1,6 hexane diol diacrylate and NVP imparted exceptionally high swelling characteristics in diverse solvents. These 2 key factors increased the efficiency of the reaction between the resin-bound and solvent-medium reagents, expanding the peptide chain. All peptides were synthesized with great purity, as demonstrated in the HPLC analysis of crude peptide and each step of the reaction was completed in an admirable way.

Materials and methods

Synthesis of PS-NVP-HDDA support

Inhibitors were removed from styrene by first washing using 1 % NaOH solution (2×30 mL) and then with distilled water (3×30 mL). The water content was removed by drying over anhydrous calcium chloride. Vacuum distillation was performed by a 4-necked reaction vessel. A thermostat, water condenser, N_2 inlet, and a Teflon-bladed stirrer, are connected to the reaction vessel. Mixture of AIBN (250 mg), NVP (0.53

mL), HDDA (0.68 mL), and styrene (10.50 mL), was mixed with a solution containing di-sodium-hydrogen phosphate (20 mg), sodium sulphate (15 g), and magnesium hydroxide (2 g), 200 mL water by stirring the solution on 1,600 rpm. Temperature of this reaction mixture was kept on 75 °C provided with a slow nitrogen stream. After 6 h, copolymers were obtained as a 200 - 400 μ sized beads. The obtained beads were purified by using water, methanol and acetone in Soxhlet extractor. The IR spectrum was obtained which clearly shows aromatic peaks and ester, 755 cm^{-1} (aromatic and 690); and 1686 cm^{-1} (ester);

Synthesis of Chloromethyl PS-NVP-HDDA support

In 50 mL DCM, the PS-NVP-HDDA (4g) was support swollen. With chloromethyl methyl ether (CMME, 24 mL) and 1 M $ZnCl_2$ in THF (0.6 mL) for 2 h at 50 °C the swollen resins were shaken, after which using a sintered glass funnel these were filtered out. These filtered resins were then washed using methanol (3×30 mL), THF/water (1:1) (3×30 mL), THF (3×30 mL), THF (4×30 mL), and then Soxhlet extractedwith methanol and THF.

Aminomethylation

In DMF, the PS-NVP-HDDA (0.24 mmol Cl, 1 g) was left for swelling and then extra DMF was discarded. 1 mL DMF is added to the resin mixed with Potassium phthalimide (0.44 g. 2.4 mmol) after which the mixture was stirred for 12 h at 120 °C. The resins were washed after filtering out with ether, DCM, THF, and DMF (all of 5×15 mL quantity). The resin was dried thoroughly dried under vacuum. The resins were swelled in distilled ethanol (20 mL) for 1 h and after which 5 % hydrazine hydrate (0.02 mL) was mixed with ethanol and this reaction mixture was refluxed on 80 °C for 8 h. These resins were washed after filtering out with ether (5×15 mL), ethanol (5×15 mL), and hot methanol (5×15 mL), then under vacuum were then dried.

PS-NVP-HDDA-HMPA support

PS-NVP-HDDA-HMPA support was synthesized using 4-Hydroxymethyl Phenoxyacetic Acid (HMPA) respectively by reacting with HOBt (2.1 g, 20 mmol) and DCC (2.5 g, 10 mmol) dissolved in DCM (12 mL) and for 1 h it was shaken. This precipitated DCU was then filtered HOBt active ester of HMPA was separated from DCM. The aminomethyl resin (5 g, 0.22 mmol NH/g) were swelled in NMP for 1 h and the extra NMP was removed by filtration. To this swollen resin the HOBt active ester of HMPA was added. After 1h these resins were filtered out and were washed with MeOH (3×30 mL), dioxane (3×30 mL), dioxane: H_2O (1:1) (3×30 mL), NMP (3×30 mL), and in vacuum were then dried. These resins were rested to have a hydroxyl capacity of 0.16 mmol OH/g. IR(KBr): 3,400 cm^{-1} (NH), 3,380 cm^{-1} (OH), 1,164 cm^{-1} (ether), 1,643 cm^{-1} (NHCO).

Peptide synthesis method using fmoc-amino acids

Manual peptide synthesizer was used to synthesize various peptides on matching swelling HMPA resins that had C-terminal amino acids attached. With 20 % piperidine solution the Fmoc protection was removed in DMF (25 mL×20 min). These resins were then washed using DMF (3×25 mL). With a combination of DIEA (3.5 meq), HOBt (7 meq), and HBTU (3.5 meq), in DMF, the coupling reactions were conducted for 50 min using suitable amino acids and then it was washed using DMF (3×20 mL). Kaiser semi-quantitative ninhydrin test was used to track the Fmoc protection cleavage as well as the degree of coupling in every cycle. Each amino acid residue was introduced by using following steps: (i) washed using DMF (4×25 mL), (ii) washed using 20 % piperidine in DMF (1×25 mL), (iii) de-protected using 20% piperidine in DMF (1×25 mL×20 min), and washed using DMF (4×25 mL).

The Fmoc protection of the N-terminal amino acid in peptide resin was eliminated following the inclusion of all amino acids (25 mL×20 min) using a 20 % piperidine solution in DMF. Resins were then dried under vacuum after they are washed with ether (5×25 mL), isopropanol (5×25 mL), and DMF (5×25 mL).

Synthesis of modularly modified fragment of E2/NS1 region of hepatitis c viral polyprotein

In a septum stoppered flask, the PS-NVP-HDDA-HMPA (500 mg, 0.054 mmol) were swelled in 10 mL DCM for 1 h. All of the extra DCM was then removed. Methyl imidazole (12.9 μL, 0.162 mmol), MSNT (48.1 mg, 0.162 mmol), Fmoc-Ala-OH (0.162 mmol, 50.43 mg), and C-terminal amino acid, were mixed in minimal quantity "of dry DCM and were added to a HMPA linker with a PS-NVP-HDDA support". This reaction mixture under nitrogen atmosphere was kept for 30 min under room temperature conditions. With DCM, ether (5×10 mL), and methanol (5×10 mL), the resins were washed thoroughly and under vacuum these were dried. Amino capacity hence was calculated to be 0.107 mmol/g. In a manual peptide synthesiser, Fmoc-Arg-HMPA-PS-NVP-HDDA (450 mg, 0.048 mmol) was taken and for it was

swelled in DMF. With 20 % piperidine, the Fmoc protection was removed in DMF (30 min; 10 mL), and was thoroughly washed with DMF (5×10 mL). For a target sequence the remaining amino acids IIe (50.88 mg, 0.144 moles), Phe (55.75 mg, 0.144 moles), Thr (56.64 mg, 0.144 moles), Tyr (66.17 mg, 0.144 moles), His (89.23 mg, 0.144 moles), Asn (86 mg, 0.144 moles), Gly (61.26 mg, 0.144 moles), Cys (84.3 mg, 0.144 moles), Pro (48.58 mg, 0.144 moles), Gly (61.26 mg, 0.144 moles), Ala (53.47 mg, 0.144 moles), Asp (59.25 mg, 0.144 moles), Leu (50.88 mg, 0.144), Arg (87.6 mg, 0.144 moles), Lys (53.05 mg, 0.144 moles).

IIe-Phe-Thr-Tyr-His-Phe-Asn-Ser-Ser-Ser-Gly-Cys(Acm)-Pro-Glu-Ala-Asp-Leu-Asp-Cys(Acm)-Arg-Ser-Lys-Leu-Arg were incorporated successively in presence of DIEA (25.08 µL, 0.144 mmol), HOBt (19.4 mg, 0.144 mmol) and HBTU (0.144 mmol, 54.35 mg). Acylation reactions were conducted twice on regions which were discovered to be positive through the ninhydrin test. The deprotection and coupling steps were examined with the ninhydrin test. Resins were washed using ether (5×10 mL), methanol (5×10 mL), DMF (5×10 mL), and under vacuum conditions were dried. The peptide were subjected to HPLC analysis by inserting them in small amount in Buffer A-C:18 RPC and were eluted with Buffer A gradient: 0.5 % TFA with water and Buffer B: 0.5 % TFA with MeCN.

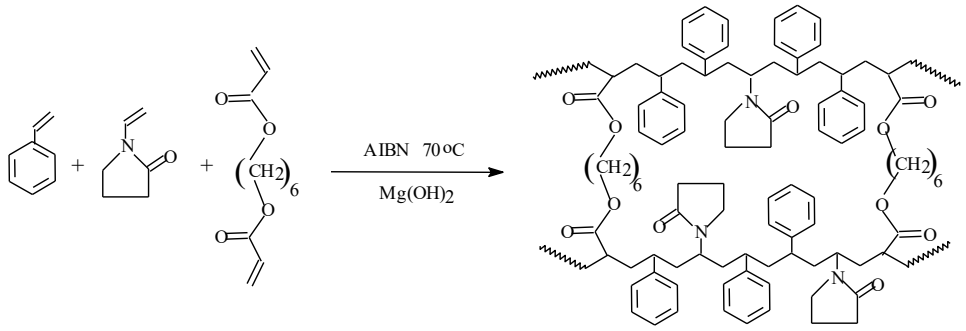
Removing peptide from polymer support

Peptides were removed from the resin by following procedure. Peptidyl resins were suspended in reagent K (phenol (200 L), ethanedithiol (150 L), thioanisole (150 L), TFA (3 mL) and water (150 L) for 4 h under room temperature conditions. At this condition the target peptides were then released from the polymer supports. The solution was filtered, and under low pressure and filtered solution was concentrated. Peptides were precipitated by adding ice-cold ether. This precipitate was then washed using ether until scavengers were removed and the sample was dried. After dissolving in water, the peptide was frozen and lyophilized.

Results and discussion

Polymer synthesis

Crosslinked polymers were made with free radical copolymerization of monomers styrene, N-vinylpyrrolidone, and 1,6-hexanediol diacrylate in an aqueous suspension (**Scheme 1**). Specific polymer percentage was made by carefully adjusting the mole ratios of the monomers. The suspension medium was created using sodium sulphate and magnesium hydroxide. Homogeneous droplets of dispersed monomer mixture floating in non-solvent phase were made using mechanical stirring. Azo bis isobutyronitrile was used as the radical initiator for the polymerization reaction. It became soluble in the droplets of monomer and aided in the thermally induced polymerization process. Temperature was increased to 70 °C before beginning the polymerization process, and was maintained until the polymerization was finished. The velocity of stirring was fixed 1,600 rpm.The shape of the reaction vessel and the quantity of the stabilizer had a profound impact on the bead size distribution of the polymer. Reproducible droplet of the monomers with 200 - 400 µ size was obtained by adjusting the stirring speed between 1,500 - 2,000 rpm. 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 1 PS-NVP-HDDA polymer synthesis.

The terpolymer has typical polystyrene peaks, the IR spectra (KBr) of powdered polymers revealed a sharp peak on 1,724 cm^{-1} which corresponds to the ester peak of the crosslinker and 1,686 cm^{-1} to carbonyl peak of NVP. A, weak peak on 148.403 ppm corresponds to the styrene C-3 and strong peak on 130.435

ppm, corresponds to aromatic polystyrene carbons appeared in the solid state ^{13}C NMR spectra. The carbonyl carbon of PVP shows up as a peak on 178.584 ppm", while the methylene carbon of a crosslinker shows up in the form of peaks at 66.437 ppm. The polymer's backbone methylene carbon is responsible for the peak at 43.548 ppm, whereas the peak at 34.465 ppm was caused by the overlapping the main carbon chain with the ring.

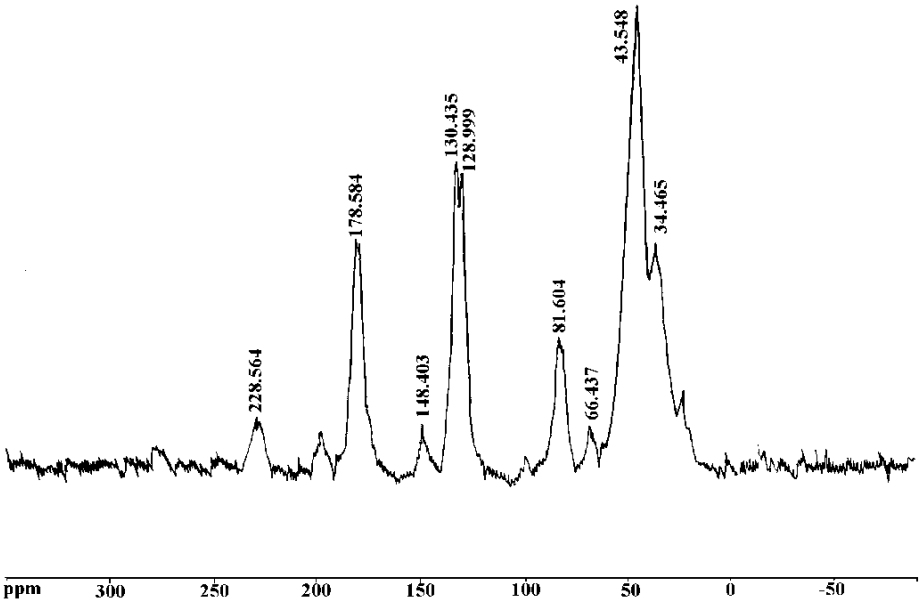


Figure 1 C-NMR[13] of PS-NVP-HDDA polymer support.

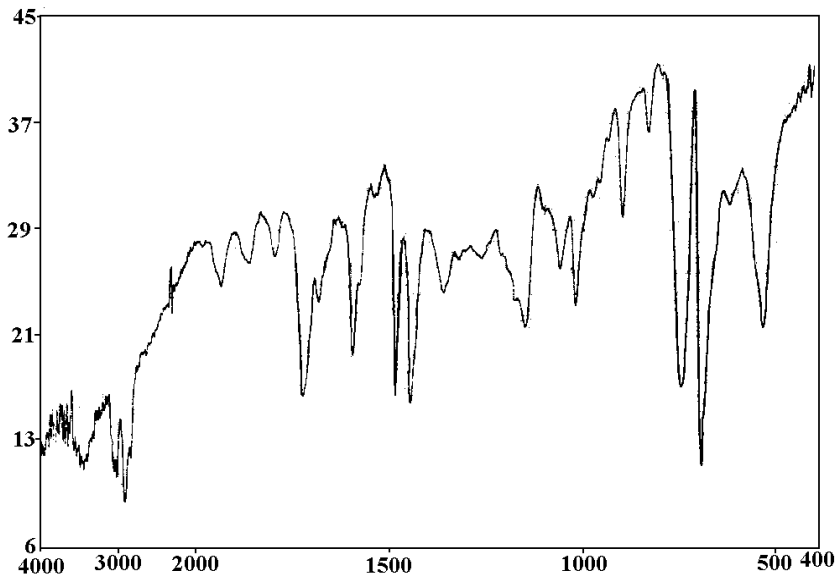


Figure 2 IR spectrum (KBr) of PS-NVP-HDDA polymer support.

SEM analysis was used to assess the polymer's morphological characteristics (**Figure 3**). It was discovered that surface of the polymer was smooth, round and unbroken. As shown by SEM of a functionalized bead, polymer morphological character did not change with the functionalization of a polymer with CMME and other chemicals. It was observed that the smoothness of the polymer surface was unchanged. The polymer amino methylation using potassium phthalimide had no impact on the polymer's morphological characteristics.

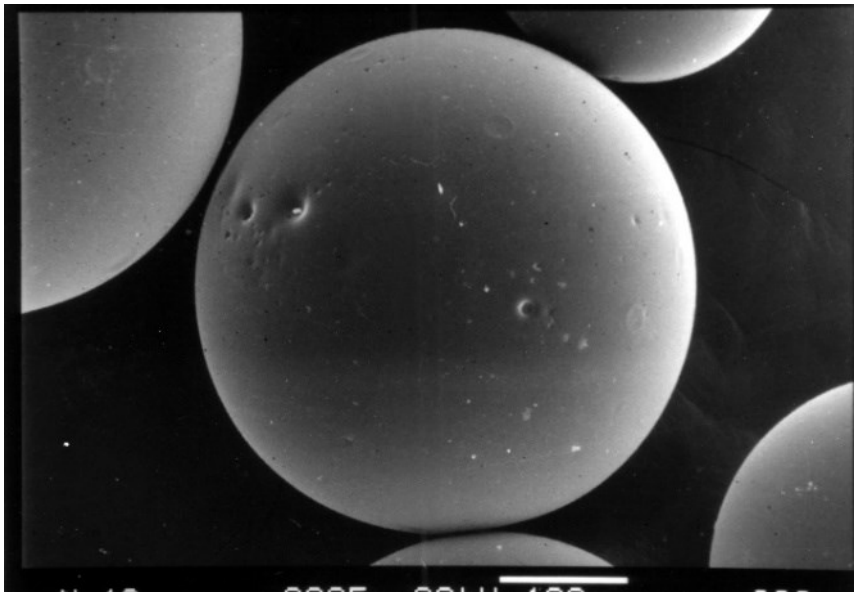
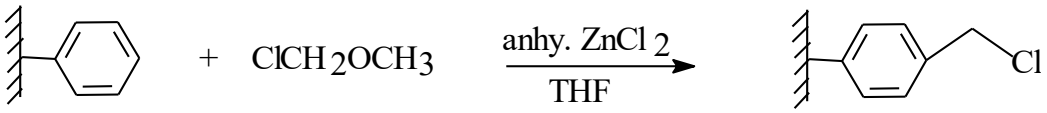


Figure 3 SEM image of PS-NVP-HDDA polymer.

The polymer was extremely stable to all reaction conditions encountered during the polypeptide synthesis. The ester bonds present in the crosslinker was found to be stable enough to withstand the strong nucleophilic attack by the bases or acids. This was investigated by suspending the polymer at 40 °C in various reagents like piperidine: DMF (1:4) mixture, 2M aqueous NaOH, 2MNH_4OH in aqueous MeOH, liquor ammonia and in neat TFA.the IR Spectrum taken after the treatment showed no significant change in the spectral characteristics.

Chloromethylation of the resin

Chloromethylation of theresin was carried out utilizing chloromethyl methyl ether (CMME) (**Scheme 2**). The method outlined in the literature was used to prepare CMME [8,9]. By utilizing $ZnCl_2$ as the Lewis acid catalyst in a Friedel-Craft type electrophilic substitution process, a chloromethyl group was added to aromatic ring of a resin. Chloromethylation proceeded smoothly when the catalyst, anhydrous $ZnCl_2$/THF was used. The Volhardt approach was used to estimate the degree of functionalization.[10]The quantity of CMME, concentration of the anhydrous $ZnCl_2$, reaction duration, and reaction medium temperature decides the extent of chloromethylation. Chloromethyl resin's IR (KBr) spectra revealed a band at 670, 1,250 and 1,420 cm^{-1} for H-C-Cl vibration and C-Cl stretch, respectively.

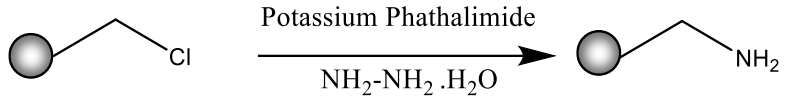


Scheme 2 Resin chloromethylation.

Chloromethylation of the polymer with low concentration of NVP using anhydrous $ZnCl_2$/THF catalyst was very effective for the controlled functionalisation reaction. The extent of chloromethylation depends upon the amount of CMME, concentration of anhydrous $ZnCl_2$, time and temperature of the reaction medium. Beyond 55 °C, the degree of chloromethylation of the resin with time, using CMME, was found to be decreasing. It might be due to the vapourisation of the CMME at higher temperature.

Aminomethylation

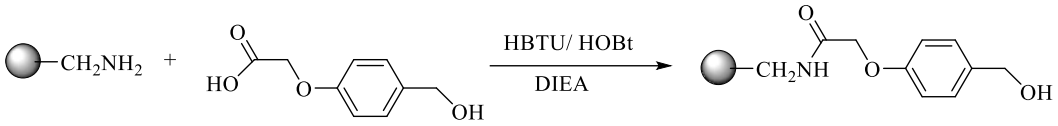
By using potassium phthalimide for Gabriel's phthalimide reaction together with hydrazinolysis, chloromethyl resin was transformed into aminoethyl resin [10]. The picric acid technique was used to gauge the degree of conversion. The assessment of amino capacity revealed that this was a quantitative conversion. The polymer's IR spectra (KBr) revealed an amino group-corresponding absorption on 3,400 cm^{-1}. A functional support is well suitable for polypeptide synthesis.



Scheme 3 Resin Aminomethylation.

Linker incorporation to resin

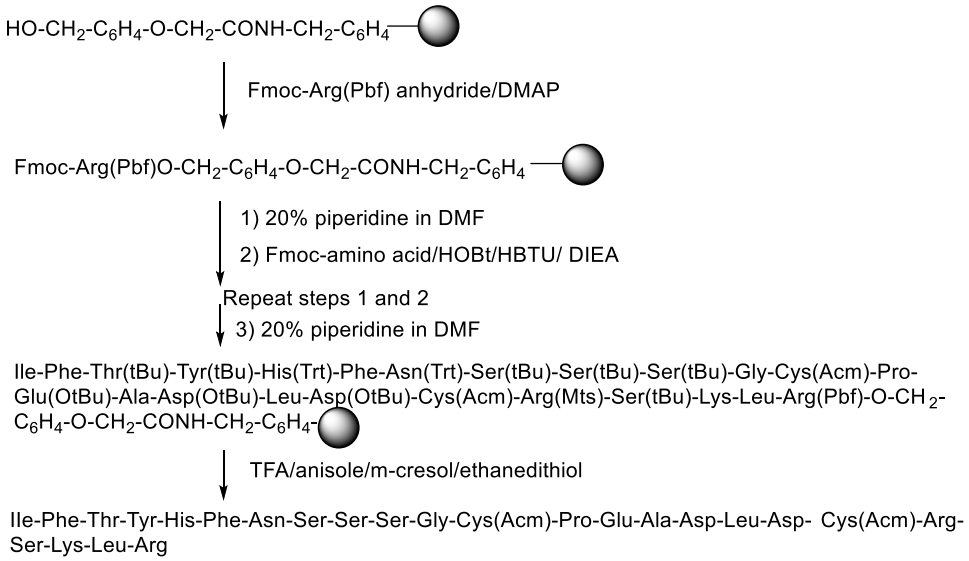
The final cleavage of the target peptide in PS-NVP-HDDA resin can be aided by introducing a particular linker or handles between the functionality of a resin and carboxylic group of C-terminal amino acid [11]. Additionally, it aids in the generation of peptides that are either protected peptides or their derivatives. Although the handle in PS-DVB resin acts as a spacer to help the site of reaction to keep away the hydrophobic environment, its function in PS-NVP-HDDA resin was restricted to speeding up the cleavage target peptide because of its hydrophilic makeup [12]. The linker, 4-Hydroxymethyl phenoxyacetic acid (HMPA) was incorporated in PS-NVP-HDDA resin by treating "H_2N-CH_2-PS-NVP-HDDA resin with HOBt active ester of HMPA".



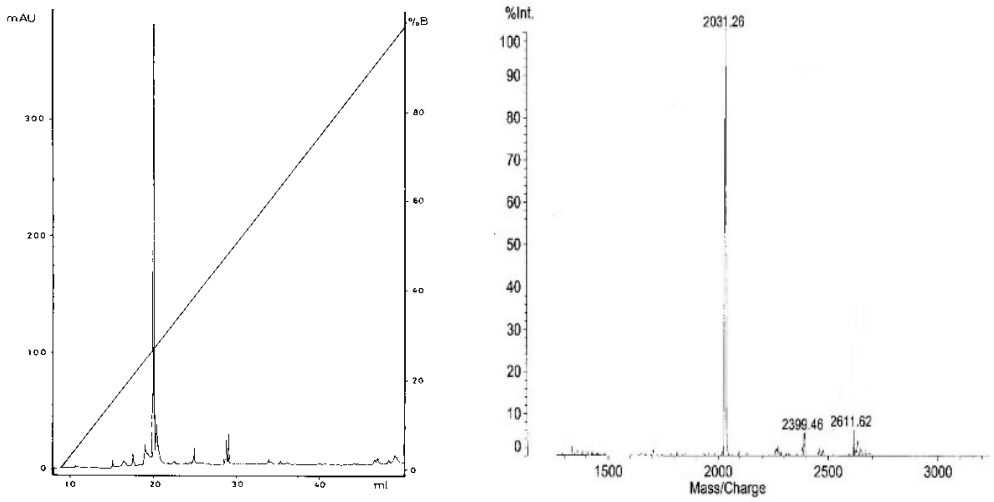
Scheme 4 Preparation of HMPA-PS-NVP-HDDA resin.

Synthesis of NS1 analogue from the non-structural region-1 of HCV polyprotein

Hepatitis is a disease characterised by inflammation of the liver, producing swelling and in many cases, cirrhosis and hepatocellular carcinoma. Hepatitis C is an RNA virus belonging to the family Flavi viride with a genome of < 9.5 kb, encoding a single polyprotein of 3010 amino acids which are subsequently spliced into ten functional protein units that were coded as: core (C), 2 envelope (E1 and E2/NS1) and atleast 6 nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). Here we attempt to synthesise modularly modified analogs of E2/NS1 peptides. The synthesis was carried out by the Fmoc strategy. Using MSNT, PS-NVP-HDDA-HMPA is attached with C-terminal Fmoc-Arg–OH. By using the 20 % piperidine in DMF, his Fmoc group was removed. Successive reactions of amino acid coupling were conducted with 3 equivalent excesses of respective Fmoc-amino acid (w.r.t. Arg load): DIEA, HBTU and HOBt. Semi-quantitative ninhydrin test was conducted to monitor all of the coupling reactions. A target peptide after synthesis was cleaved from a support using TFA when acid scavengers are present like ethanedithiol, *m*-cresol and anisole (**Scheme 5**). An 80 % yield was obtained in the case of crude peptide. The resultant white powder was dissolved in water, it was then deep frozen and lyophilised.



Scheme 5 Synthesis of IIe-Phe-Thr-Tyr-His-Phe-Asn-Ser-Ser-Ser-Gly-Cys(Acm)-Pro-Glu-Ala-Asp-Leu-Asp- Cys(Acm)-Arg-Ser-Lys-Leu-Arg: NS1 peptide analogue synthesis on PS-NVP-HDDA-HMPA resin.



(a) (b)

Figure 4 (a) HPLC analysis of E2/NS1 peptide. Buffer A: 0.1 % TFA in water, Buffer B: 0.08 % TFA in 80 % MeCN in water. Rate of Flow: 1 mL/min. Gradient: 0 - 100 % B in 50 min. (b) MALDI-TOF-MS of E2/NS1 analogue.

Analytical HPLC of the crude peptide showed only 1 major peak and MALDI TOF MS analysis showed that this peak is corresponding to the target peptide (**Figure 7**). The peptide if further purified by using preparative HPLC for further analysis. MALDI-TOF-MS m/z 2041.9 Da[$(M+H)^+$, 100 %]," $C_{86}H_{134}N_{29}O_{30}S_1$ requires M^+ 2039 Da (**Figure 4(b)**). Analysis of Amino acid: Ser, 1.69(2); Gly, 0.98(1); Ile, 1.01(1); His, 0.92(1); Arg, 0.98(1); Asp, 5.01(5); Leu, 2(2); Thr, 0.75(1); Ala, 2(2); Cys, 0.98(1). During hydrolysis, Trp was destroyed.

Conclusions

The crosslinked terpolymer PS-NVP-HDDA, was synthesised in the bead form by the radical aqueous suspension polymerization of styrene, 1,6-hexanediol diacrylate and N-vinylpyrrolidone (NVP) is used as an effective solid support for the solid phase polypeptide synthesis. This resin showed excellent mechanical stability, and a hydrophobic-hydrophilic balance required for a good polymer support. High swelling nature of the polymer in all solvents used for the chemical synthesis of the polypeptide facilitated the free interaction of the resin-bound functional sites and the reagents caused enhanced rate of amide bond formation. The utility of the terpolymer was proved beyond doubt by the synthesis of modularly modified analogs of E2/NS1 fraction of the acyl carrier protein. The peptide formed has excellent yield and purity revealed by the HPLC and MALDI.

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