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Synthesis of Biologically Active Peptides Using Newly Designed N-Vinyl Pyrrolidone Incorporated Flexible Crosslinked Polystyrene

SUNIL JACOB[1]*, NISSY ANN HARRY[1], K. K. BINOJ[1], PREETHY SOOSAN THOMAS[1] and JANEY MARY MATHEW[1]

[1]Department of Chemistry, Catholicate College, Pathanamthitta. Kerala 689645, India.
*Corresponding author E-mail: suniljacob07@gmail.com

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ABSTRACT

Synthesis of NS1 and NS2 peptide fragment of hepatitis C virus polyprotein was carried out on a terpolymer- polystyrene, N-vinylpyrrolidone, 1,6-hexanediol diacrylate resin (PS-NVP-HDDA). The radical aqueous suspension polymerisation resulted in the resin, that exhibited high swelling capacity in various solvents. The peptides were synthesized step-wise by Fmoc strategy, established with amino acid analysis and purified using HPLC. The polymer's high degree of swelling might facilitate free reagent interaction with resin-bound functional sites, resulting in an increased rate of amide bond production. The peptides yield and purity obtained from new support was high when compared to Merrifield resin. These peptides synthesis depicts the application of PS-NVP-HDDA resin developed for synthesis of long chain peptides in high homogeneity and high yield.

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

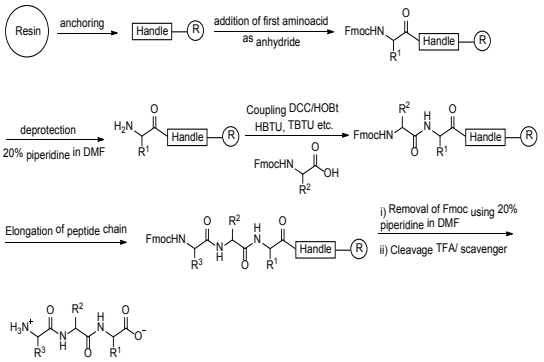
INTRODUCTION

Choosing a 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. Merrifield made a scientific discovery in 1963 that helped spark a boom in polymer-supported organic synthesis decades later.[1] Merrifield resin, a polymer of divinyl benzene and polystyrene that is only weakly crosslinked and as the attachment point has a pendant chloromethyl group. The rigid nature of the polymer, insufficient solvation in polar organic solvents, nonlinear kinetic behavior caused by irregular distributions, inaccessibility of functional areas hidden in the polymer core to the substrates, and similar issues related to heterogeneous reaction conditions resulted in truncated and deletion sequence.[2] Polarities of supports must be similar to the polarities of the chemicals used, the solvents, and resin-bound peptide chains used for a solid-phase reaction to be successful.[3] Much work has been given to creating new polymeric supports with enhanced polarity in comparison with Merrifield resin in order to advance solid-phase peptide synthesis, which include “polyamide-based supports, Polyoxyethylene-

Polyoxypropylene (POEPOP), poly (ethylene glycol)-poly-(Acrylamide) (PEGA), poly(ethylene glycol)–polystyrene (PS) graft resins, polyoxyethylene-polystyrene (POEPS), crosslinked ethoxylate acrylate resin (CLEAR), super permeable organic combinatorial chemistry resin (SPOCC), JandaJel, hydroxy- and amine-functionalized resin (HYDRA), and crosslinked polystyrene-ethylene glycol acrylate resin (CLPSER).[4] Series of new polymeric supports based on styrene were designed in our laboratories with radical aqueous suspension copolymerization of respective monomers, which introduces flexible hydrophilic crosslinkers to polystyrene. These polymers are Butanediol Dimethacrylate Cross-linked Polystyrene (PS-BDODMA), Tetraethylene Glycol Diacrylate Crosslinked Polystyrene (PS-TTEGDA), and 1,6-Hexanediol Diacrylate Crosslinked Polystyrene (PS-HDDA).[5]

Hepatitis is a condition which occurs due to liver inflammation, it results in swelling and also in numerous cases hepatocellular carcinoma or cirrhosis. Hepatitis C is categorized under RNA virus of Flaviviridae family with <9.5kb genome, encoding single poly-protein of 3010-amino acids spliced subsequently in 10-units of functional proteins coded as: "NS2, NS3, NS4A, NS4B, NS5A and NS5B" the 6 non-structural proteins; E1 and E2/NS1 the two envelope proteins and the core (C) protein.[6]

Serological HCV detection was seriously hampered by the HCV genome's variability. In most cases, the detection was performed using an ultra-sensitive PCR (Polymerase Chain Reaction). However, this method requires proper training and is also quite expensive.[7] Owing to these two 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 H-GSWHINRTALNCNDSLNTA-OH". The present study is cantered on the synthesis of 19 residual fragments of E2/NS1 region and twenty-five peptide residues designed from the protein fraction NS2 of hepatitis C virus polyprotein on the newly developed terpolymer polystyrene, N-vinylpyrrolidone, 1,6-hexanediol diacrylate resin (PS-NVP-HDDA) developed by radical aqueous suspension polymerization of styrene, 1,6-hexanediol, N-vinylpyrrolidone (NVP), diacrylate its usage in peptide solid-phase synthesis. These resins displayed excellent mechanical stability, swelling characteristics, and a hydrophobic-hydrophilic balance. The 1,6-hexanediol diacrylate and hydrophilic N-vinylpyrrolidone monomer crosslinker present in polymer gave rise to exceptionally high swelling characteristics in diverse solvents. This characteristic increased the efficiency of the reaction between the resin-bound and solvent-medium reagents, expanding the peptide chain. As every step of this reaction was completed to get new raisin, all peptides were synthesized with great purity, as demonstrated in the HPLC analysis of crude peptide.



Scheme 1. Peptides step-wise synthesis using Fmoc strategy on linker incorporated PS-NVP-HDDA resin

EXPERIMENTAL

MATERIALS AND METHODS

MSNT, Boc and Fmoc-amino acids, HOBt, HMPA, "2-(1H-benzotriazol-1-yl)1,1,3,3-tetramethyluroniumhexafluorophosphate" (HBTU), dicyclohexyl carbodiimide (DCC), Sheppard resins (Novasyn® KA 125), cesium carbonate, 4-(Dimethylamino) pyridine (DMAP), and Styrene, all "were purchased from Novabiochem Limited, UK; piperidine, 4-(hydroxymethyl) 3-(methoxy) phenoxy butyric acid (HMPB)", diisopropylethylamine (DIEA), TFA, ethanedithiol, thioanisole, 1,6-hexanediol diacrylate (HDDA), were bought "from Sigma-Aldrich Corporation, USA. N-Vinylpyrrolidone and 1,6-Hexanediol diacrylate were bought from E. Merck, Germany". A literature method was followed for synthesizing Chloromethylmethyl Ether (CMME).[8] "Solvents of HPLC grade were bought from E. Merck (India) and BDH (India). A Bruker 300-MSL tool operating on 75.47MHz was used to measure the ^{13}C NMR measurements. IR spectra was obtained

on a Shimadzu IR 470 spectrometer using KBr pellets." C-18 semi-preparative reverse phase HPLC column was used to perform HPLC on a Pharmacia instrument. Amino acid analyzer- LKB 4151 Alpha plus was employed to conduct amino acid analysis. A peptide using 6N HCl was hydrolyzed in a Pyrex glass tube for 15 h, under N_2 at 130°C for this purpose.

Synthesis of PS-NVP-HDDA support

We removed inhibitors from styrene by first washing using 1%NaOH solution (2×30 mL) and then with distilled water (3×30 mL). This is then dried over anhydrous calcium chloride. Vacuum distillation ensured the purification of NVP. A water condenser, a thermostat, N_2 inlet, and a Teflon-bladed stirrer, are connected to a Four-necked reaction vessel. Mixture of AIBN (200 mg), NVP (0.54 mL), HDDA (0.67 mL), and styrene (10.54 mL), was mixed with a solution containing di-sodium-hydrogen phosphate (10 mg), sodium sulphate (10 g), and magnesium hydroxide (1 g), 100 mL water by stirring the solution on 1600rpm. Temperature of this reaction mixture was kept on 70°C provided with a slow nitrogen stream. After 6 h , copolymers were obtained as a 200-400μ sized beads. Using hot water, the polymers were washed for removing stabiliser, methanol (3×50 mL), and acetone (3×50 mL), which were purified further by using acetone methanol in Soxhlet extractor it was then under vacuum dried thoroughly. The IR spectrum was obtained which clearly shows ester and aromatic peaks, 690 and 755 cm^{-1} (aromatic); 1686 cm^{-1} (ester); and IR (KBr): 1724.

Synthesis of Chloromethyl PS-NVP-HDDA support

In 50 mL DCM, the PS-NVP-HDDA (4 g) was support swollen. Extra DCM after one hour was filtered out. With chloromethyl methyl ether (CMME,24 mL) and 1M $ZnCl_2$ 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 (0.24mmol Cl, 1 g) was left for swelling and then extra DMF was discarded. The 1 mL DMF added to the resin mixed with Potassium phthalimide (0.44g. 2.4mmol) 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 5x15 mL quantity), and under vacuum were thoroughly dried. The resins were swollen for 1 h in distilled ethanol (20 mL) after which 5% hydrazine hydrate (0.02 mL) was mixed with ethanol and this reaction mixture for 8 h was refluxed on 80°C. These resins were washed after filtering out with ether (5x15 mL), ethanol (5x15 mL), and hot methanol (5x15 mL), then under vacuum were then dried.

Swelling behavior

1 g of resin was placed on a syringe filled of sintered Teflon filter, and from top of this syringe this solvent was added. A steady suction was applied at the outlet of a syringe produced the solvent flow. One millilitre per minute of flow was achieved by controlling the suction. For 30 min this solvent was made to pass through resin. These resins were suspended in a solvent for one hour with the syringe exit closed. The syringe's piston compressed the swelled resin, and then the pressure was gradually released. In order to assess the resin's capacity for swelling, resin volume on this stage was measured and compared with the weight of the sample. This same experiment was used for PS-DVB resin. The increase in weight of solvent-swollen resins were compared to the dry sample resins.

PS-NVP-HDDA-HMPA and PS-NVP-HDDA-HMPB support

PS-NVP-HDDA-HMPB and PS-NVP-HDDA-HMPA support was synthesized using HMPB Acid (1.89 g, 10mmol) and 4-Hydroxymethyl Phenoxyacetic acid (HMPA) respectively by reacting with DCC (2 g, 10mmol) and HOBt (2.2 g, 20mmol) dissolved in DCM (10 mL) and for one hour it was shaken. This precipitated DCU was then filtered. In vacuum this DCM was removed from a filtrate which resulted in HOBt active ester of HMPA was dried in vacuum. In an NMP solvent (100 mL) the Aminomethyl resin (5 g, 0.24mmol NH/g) were swelled for one hour and the extra NMP was removed with filtration. To this swollen resin the HOBt active ester of HMPB/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 0.16mmol OH/g. IR(KBr): 3400 cm^{-1} (NH), 3380 cm^{-1} (OH), 1164 cm^{-1} (ether), 1643 cm^{-1} (NHCO).

Peptide Synthesis Method using Fmoc-amino acids

A manual peptide synthesizer was used to synthesize various peptides on matching swelling HMPA and HMPB resins that had C-terminal amino acids attached. With 20% piperidine solution the Fmoc protection was removed in DMF (25 mL×20 minute). These resins were then washed using DMF (3×25 mL). With a combination of DIEA (3.5meq), HOBt (7meq), and HBTU (3.5meq), in DMF, the coupling reactions were conducted for 50 min using suitable amino acids (3.5meq excess in terms of the capacity of C-terminal amino acid with HMPB resin attached) and then using DMF (3×20 mL) it was washed. 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).

In order to acylate the C-terminal amino acid present in HMPB resin, HBTU, 3.5mmol more Fmoc-amino acid, DIEA, and 7mmol more HOBt were used. The Fmoc protection of the N-terminal amino acid in peptide resin was eliminated following the inclusion of all amino acids (25 mLx20 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 19-residue fragment of E2/NS1 region of Hepatitis C viral polyprotein

In a septum stoppered flask, the PS-NVP-HDDA-HMPA (500mg, 0.054mmol) were swelled in 10 mL DCM for 1 hour. All of the extra DCM was then removed. Methyl imidazole (12.9 µL, 0.162mmol), MSNT (48.1 mg, 0.162mmol), Fmoc-Ala-OH (0.162mmol, 50.43mg), 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 0.107mmol/g. In a manual peptide synthesiser, Fmoc-Ala-HMPA-PS-NVP-HDDA (450mg, 0.048mmol) was taken and for ih 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 Thr (56.64mg, 0.144mmol), Gly (42.81mg, 0.144mmol), Asn (86mg, 0.144mmol), Trp (61.4mg, 0.144mmol), Leu (50.88mg, 0.144mmol), His (89.23mg, 0.144mmol), Ile (50.88mg, 0.144mmol), Ser (55.2mg, 0.144mmol), Arg (87.65mg, 0.144mmol), Cys (84.3mg, 0.144mmol), Asp (59.25mg, 0.144mmol), were incorporated successively in presence of DIEA (25.08 µL, 0.144mmol), HOBt (19.4mg, 0.144mmol) and HBTU (0.144mmol, 54.35mg). 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 underwent HPLC analysis by inserting them in small amount in Buffer A-C:18RPC 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

The following techniques were used to separate the peptides from resin. By suspending peptidyl resins Reagent K (phenol (200l), ethanedithiol (150l), thioanisole (150l), TFA (3 mL) and water (150l) for 4 h under room temperature conditions, target peptides were then released from polymer supports. This solution was filtered, and under low pressure, filtered solution was concentrated. By adding ice-cold ether, peptides were precipitated. This precipitate was then washed using ether till scavengers were filtered and dried. After dissolving in water, the peptide was frozen and lyophilized.

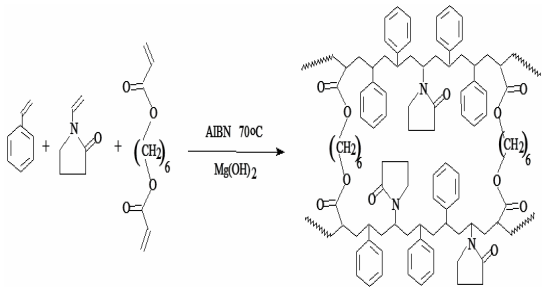
RESULTS AND DISCUSSION

Polymer Synthesis

Crosslinked polymers were made with free radical copolymerization of monomers styrene, N-vinylpyrrolidone, & 1,6-hexanediol diacrylate in an aqueous suspension. The quantity of formed monomers was chosen based on mole ratios necessary for creating specific polymer percentage. The suspension medium was supplemented with sodium sulphate and magnesium hydroxide

(Scheme 2). Small, homogeneous droplets of dispersed monomer mixture floating in non-solvent phase were made using mechanical stirring. The radical initiator AIBN was added to start, the polymerization reaction. It became soluble in the droplets of monomer and aided in the thermally induced polymerization process. Temperature level was increased to 70°C before beginning the polymerization process, and was maintained until the polymerization was finished. The velocity of stirring, reaction vessel shape, and quantity of the stabilizer were all found to have an impact on the polymer's bead size distribution.

Shape of polymerization vessel used and the shape along with speed of paddles all have an impact on the development of the droplets in which the polymerization occurs, which affects the quality and size of the beads. A non-solvent phase might be imagined as offering countless small "spherical molds" where the surface tension constrains the generated polymer beads. Stabilizers work to lower the droplet surface tension and preventing aggregation of droplets, resulting in deformed beads. The suspended mixture is pushed in the direction of the revolving stirrer blade by the indentation force caused by the shape of the vessel. This may result in a uniformly sized monomer droplets being sheared in a homogenous environment. By varying the stirring speed between 1500 and 2000rpm, reproducible droplets of the monomers with sizes between 200 and 400μ were produced. Beyond 3000rpm, it was observed that the polymer yields significantly decreased. This can be the result of the polymer bead being sheared too much. It was found that after each polymerization cycle, the subsequent reaction only continues without issue provided the reaction vessel has been salinized. The size and yield of novel PS-NVP-HDDA polymer may be replicated by carefully adjusting the aforementioned parameters.



Scheme 2. PS-NVP-HDDA polymer Synthesis

Characterization of polymer PS-NVP-HDDA was done using ^{13}C NMR and IR methods. Along with the typical polystyrene peaks, the IR spectra (KBr) of powdered polymers revealed a "sharp peak on 1724 cm^{-1} which corresponds to ester carbonyl of crosslinker and 1686 cm^{-1} to carbonyl peak of NVP. A strong peak on 130.435ppm, corresponds to aromatic polystyrene carbons, and weak peak on 148.403ppm corresponds to the styrene C-3, appeared in the solid state ^{13}C NMR spectra. The carbonyl carbon of PVP shows up as a peak on 178.584ppm", while the methylene carbon of a crosslinker shows up in the form of peaks on 66.437ppm. The polymer's backbone methylene carbon is responsible for the peak on 43.548ppm, whereas the peak on 34.465ppm was caused by overlapping the main carbon chain with the ring.

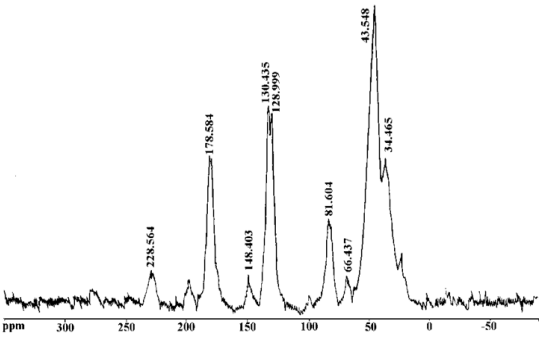


Fig. 1. ^{13}C-NMR of PS-NVP-HDDA polymer support

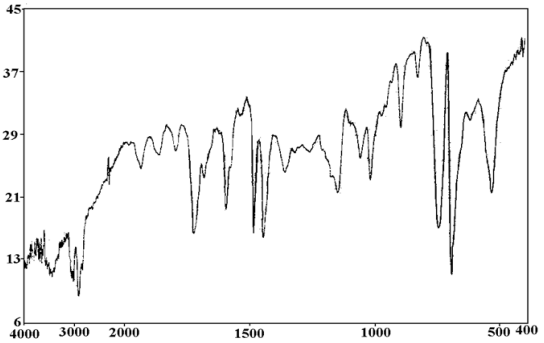


Fig. 2. IR spectrum (KBr) of PS-NVP-HDDA polymer support

SEM analysis was used to assess the polymer's morphological characteristics (Fig. 3). It was discovered that surface of the polymer was 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 aminomethylation using potassium phthalimide had no impact on the polymer's morphological characteristics.

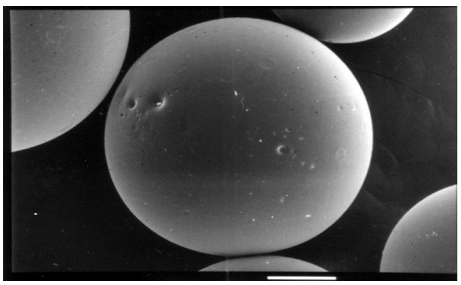


Fig. 3. SEM image of PS-NVP-HDDA polymer

Polymer stability

During polypeptide synthesis, this polymer was exceedingly stable in every reactive condition. It was found that the ester bonds in the crosslinker were sufficiently stable to resist nucleophilic attack by any acids or bases. To examine this, the polymer was suspended at 40°C in a variety of chemicals, including piperidine: 1:4 DMF combination, 2M NH_4OH, in aqueous MeOH, 2M aqueous NaOH, neat TFA, and liquid ammonia. IR spectrum (KBr) of a resin was taken both pre and post treatment with appropriate chemicals, all of the resins were dried after washing, after which the chemical integrity was assessed of the polymer. The spectrum revealed no changes in their chemical composition relative to the initial spectrum, indicating that this support is sufficiently stable for enduring every condition involved in the synthesis of peptides (Figure 4).

After the short peptide sequences are produced on a resin and its cleavage, the stability of this resin was once again examined. Resins were then cleaned using different solvents after being removed from the cleavage solution, and it was then powdered after drying it and was pelletized with KBr. Infrared spectrum results were same as those of the original resin. Therefore, the IR spectra (KBr) of resin obtained following treatment using several chemicals employed for peptide synthesis demonstrated that chemical character of the polymer has not changed.

Resin functionalization

Chloromethylation of the resin

Chloromethyl functionalization was employed in the early stages of the creation of the new resin, and it was carried out utilizing chloromethyl methyl ether (CMME) (Scheme 3). The method outlined in the literature was used to prepare CMME.[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. Anhydrous $SnCl_2$ exhibited extremely high catalytic activity. Since the anhydrous $SnCl_2$ reaction happened so quickly, it was exceedingly challenging to functionalize the resin under regulated conditions using this catalyst. Using $SnCl_2$ for chloromethylation resulted in altering the color of resins, which could result in a number of problems in the synthesis of peptide, particularly in the color sensitive ninhydrin reaction for tracking the magnitude of coupling reactions. 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 formation of Zn-NVP complex was a problem which may develop when resins were chloromethylated with high NVP content was done with anhydrous $ZnCl_2$/THF catalyst which resulted in a change in color. This changed color did not wash away even when washed with several solvents, demonstrating the excellent stability this compound has. This complex hence formed was confirmed further by complete burning of a polymer which created a yellow-colored residual in crucible as well as by the IR (KBr) spectrum. When a lower NVP percent is utilized to create the polymer, this effect can be avoided.

The controlled functionalization procedure used an anhydrous $ZnCl_2$/THF catalyst to chloromethylate the polymer with low concentrations of NVP. The quantity of CMME, anhydrous $ZnCl_2$ concentration, reaction duration, and reaction medium temperature all determined the effect of chloromethylation. It was observed that as the temperature was raised above 55°C, the resin's chloromethylation level decreased with time. It may be caused by the CMME vaporizing at a greater temperature. Chloromethyl resin's IR (KBr) spectra revealed a band at 670 cm^{-1}, 1250 cm^{-1} and 1420 cm^{-1} for H-C-Cl vibration and C-Cl stretch, respectively.

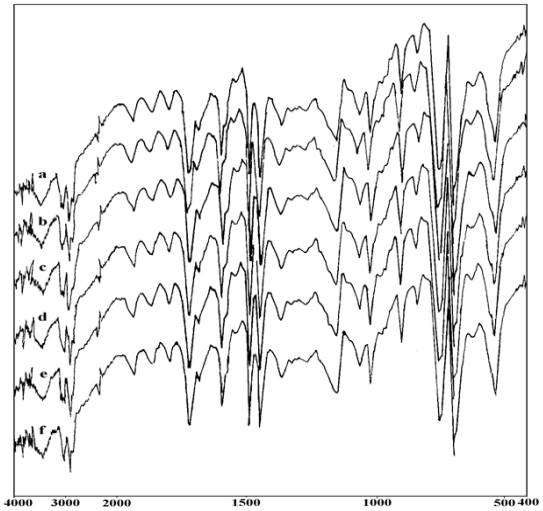


Fig. 4. "IR (KBr) spectra of PS-NVP-HDDA support, (a) original, b-f after 48 h treatment of the resin with following reagents, (b) piperidine: DMF (1:4) mixture; (c) 2 M aqueous NaOH; (d) 2 M NH2OH in aqueous MeOH; (e) liquor ammonia; (f) neat TFA"

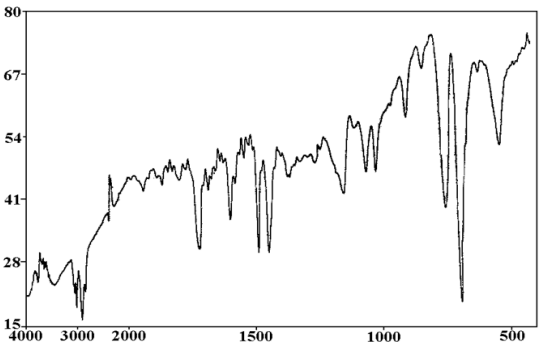
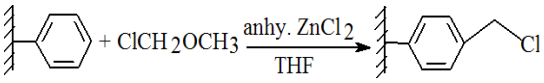


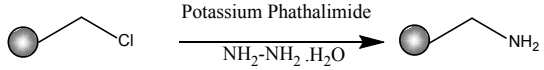
Fig. 5. "IR (KBr) spectrum of the chloromethylated PS-NVP-HDDA polymer support"



Scheme 3. Resin Chloromethylation

Aminomethylation

By using potassium phthalimide for Gabriel's phthalimide reaction together with hydrazinolysis, chloromethyl resin was transformed into aminoethyl resin. The picric acid technique was used to gauge the degree of conversion.[11] 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 3400 cm^{-1}. A functional support is well suitable for polypeptide synthesis.



Scheme 4. Resin Aminomethylation

Swelling characteristics of the resin

We examined and compared the polymer's swelling (shown in Fig. 6) with PS-DVB resin in different solvents. As per the figure, the PS-NVP-HDDA had higher swelling characteristics in all solvents. The porosity measurements of the new PS-NVP-HDDA resin revealed that it is very microporous, resulting in a huge surface area (Table 1). Solid phase reactions depend on the pace at which chemicals diffuse into the resin since they are heterogeneous processes. In various solvents, the impact of aminomethylation and chloromethylation on polymer swelling was further investigated.

Table 1: "Adsorption BJH pore volume distribution of 200-400□ PS-NVP-HDDA resin"

Diameter range(nm)	Average diameter(nm)	d(Vp)/d (Dp) (mL/g*nm)	Incremental volume (mL/g)	Cumulative volume (mL/g)	Volume%
159.13-136.94	148.13	0	0.00007	0.00007	0.47
136.94-120.13	128.53	0	0.00007	0.00014	0.47
120.13-107.05	113.59	0.00001	0.00007	0.0002	0.47
107.05-96.57	101.81	0.00001	0.00006	0.00027	0.44
96.57-77.70	87.14	0.00001	0.00017	0.00044	1.18
77.70-65.10	71.4	0.00001	0.00017	0.00061	1.15
65.10-44.01	54.55	0.00002	0.00049	0.0011	3.36
44.01-33.38	38.69	0.00005	0.00049	0.00159	3.37
33.38-26.96	30.17	0.00008	0.0005	0.00208	3.42
26.96-22.65	24.8	0.00012	0.0005	0.00258	3.44
22.65-19.54	21.09	0.00016	0.00049	0.00308	3.38
19.54-17.19	18.36	0.00021	0.00049	0.00356	3.35
17.19-14.82	16.01	0.00027	0.00064	0.0042	4.41
14.82-13.03	13.93	0.00035	0.00063	0.00483	4.31

13.03-11.62	12.33	0.00044	0.00061	0.00544	4.23
11.62-10.48	11.05	0.00053	0.00061	0.00605	4.18
10.48-9.54	10.01	0.00064	0.0006	0.00665	4.11
9.54-8.75	9.15	0.00074	0.00058	0.00723	4.02
8.75-8.08	8.42	0.00084	0.00056	0.0078	3.89
8.08-7.50	7.79	0.00096	0.00056	0.00836	3.86
7.50-6.99	7.24	0.00107	0.00055	0.00891	3.76
6.99-6.54	6.76	0.00118	0.00053	0.00944	3.67
6.54-6.14	6.34	0.0013	0.00052	0.00996	3.58
6.14-5.45	5.79	0.00144	0.00098	0.01094	6.77
5.45-4.89	5.17	0.0016	0.0009	0.01185	6.21
4.89-4.41	4.65	0.0018	0.00086	0.0127	5.89
4.41-4.20	4.3	0.00191	0.0004	0.01311	2.77
4.20-3.82	4.01	0.00195	0.00074	0.01385	5.09
3.82-3.48	3.65	0.00207	0.00069	0.01454	4.75

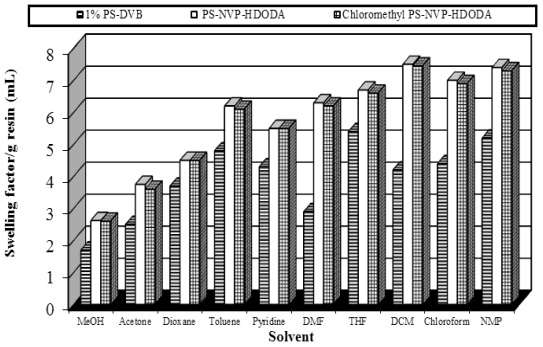
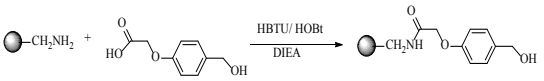


Fig. 6. Comparing Swelling of PS-NVP-HDDA and Chloromethyl -PS-NVP-HDDA with PS-DVB in different solvents

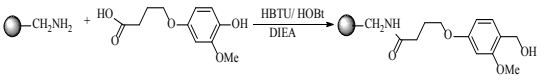
These results were compared to the PS-DVB resin (Merrifield resin). Swelling properties of the functionalized and unfunctionalized resins did not differ noticeably. But it was discovered that the nature of the solvent affected the peptidyl-tendency resins to swell. Due to the altered polarity of the polymer matrix, peptidyl resin exhibited relatively better swelling characteristics in DMF than DCM.

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. 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 linkers, 4-(Hydroxymethyl) 3-(methoxy) phenoxy butyric acid (HMPB) and 4-Hydroxymethyl phenoxyacetic acid (HMPA) were employed in PS-NVP-HDDA resin by treating "H_2N-CH_2-PS-NVP-HDDA resin with HOBt active ester of HMPA" and HMPB respectively.



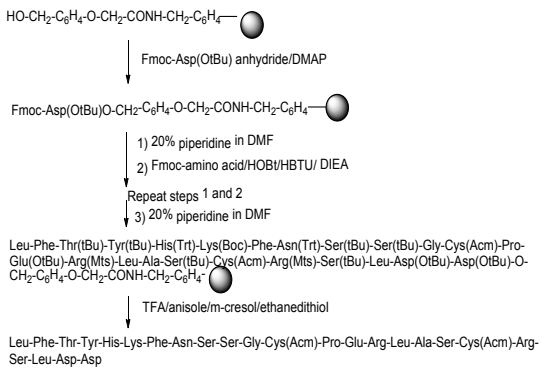
Scheme 5. Preparation of HMPA-PS-NVP-HDDA resin



Scheme 6. Preparation of HMPB-PS-NVP-HDDA resin

Peptide Synthesis designed from NS1 protein fraction of hepatitis C virus polyprotein

The synthesis was carried out by the Fmoc strategy. Using MSNT, PS-NVP-HDDA-HMPA is attached with C-terminal Fmoc-Ala–OH. By using the 20% piperidine in DMF, his Fmoc group was removed. Successive reactions of amino acid coupling were conducted with three equivalent excesses of respective Fmoc-amino acid (w.r.t. Ala 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 7). An 80% yield was obtained in the case of Crude peptide. The resultant white powder was mixed in water, it was then deep frozen and lyophilised. In analytical HPLC the major peak corresponding to target peptide (Fig. 7). "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 (Fig. 4. 12b). 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.



Scheme 7. NS1 peptide Synthesis on PS-NVP-HDDA-HMPA resin

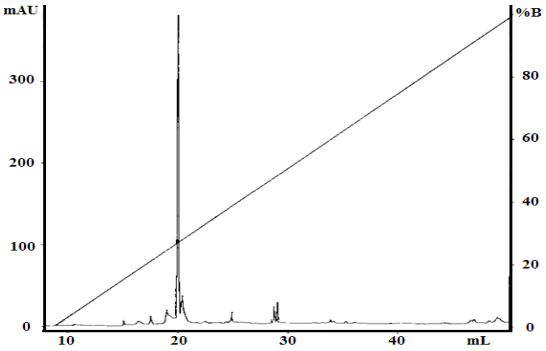
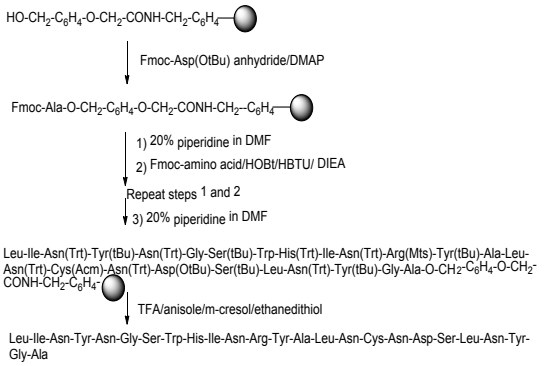


Fig. 7. "HPLC analysis of E2/NS1 peptide. Buffer A: 0.1% TFA in water, Buffer B: 0.08% TFA in 80% MeCN in water. Rate of Flow: 1 mL/minute. Gradient: 0-100 % B in 50 minute

Twenty-Five residue peptide Synthesis designed from NS2 protein fraction of hepatitis C virus polyprotein

From non-structural HCV polyprotein part, 25 residue NS2 peptide "Leu-Ile-Asn-Tyr-Asn-Gly-Ser-Trp-His-Ile-Asn-Arg-Tyr-Ala-Leu-Asn-Cys-Asn-Asp-Ser-Leu-Asn-Tyr-Gly-Ala" were synthesized on a PS-NVP-HDDA-HMPA resin. When DMAP was present, the symmetric anhydride of Fmoc-Ala was used to connect the C-terminal amino acid with a support. Complete Fmoc-Gly incorporation took place after second attempt. Following Fmoc protection removal using 20% piperidine in DMF, the appropriate amino acids were added using HOBt and HBTU with DIEA present. Semi-quantitative ninhydrin test was conducted to monitor all of the coupling reactions. Target peptide after synthesis was cleaved from a support using TFA with ethanedithiol, m cresol and anisole, present as acid scavengers (Scheme 8). Approximately 80% yield was obtained for Crude peptide. The resultant white powder was mixed in water, it was then deep frozen and lyophilized. There was only one single peak seen in the HPLC profile (Fig. 8). Data analysis of amino acid also agrees with the target peptide "A, 2.02 (2); G, 2.00 (2); T, 2.68 (3); D, 6.78 (7); L, 3.03 (3) L, 1.67 (2); C, 0.89 (1); R, 1.03 (1); I, 2.01 (2); H, 0.90 (1)". During hydrolysis the Trp was destroyed and Asn hydrolyzed into Asp. Higher Asp value was because of hydrolyzing one Asn into Asp. Ser had a low value owing to the partial hydrolysis.



Scheme 8. NS2 peptide Synthesis on PS-NVP-HDDA-HMPA resin

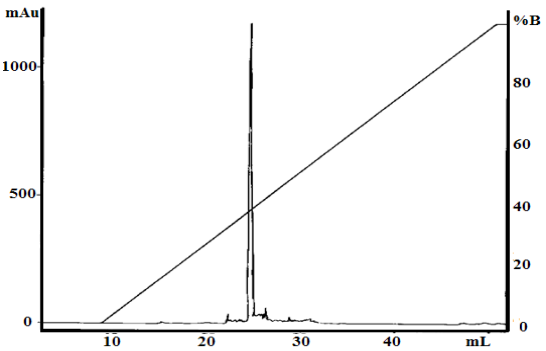


Fig. 8. "HPLC profile of 25 residue peptides designed from NS2 protein region of HCV A: 0.5% TFA in water, B: 0.5% TFA in acetonitrile, Gradient used: 0% to100% B in 40 min; Flow rate: 1 mL/minute"

CONCLUSION

Thus, the terpolymer of polystyrene, N-vinylpyrrolidone, 1,6-hexanediol diacrylate resin (PS-NVP-HDDA) was developed by the radical aqueous suspension polymerization of styrene, 1,6-hexanediol diacrylate, N-vinylpyrrolidone (NVP), its effectiveness in solid-phase peptide synthesis, was compared with the Merrifield resin. The resin displayed excellent mechanical stability, swelling characteristics, and a hydrophobic-hydrophilic balance. The effectiveness of the new polymer was then evaluated by synthesizing NS1 and NS2 peptide fragment of hepatitis C virus polyprotein. The polymer's high degree of swelling

might facilitate free reagent interaction with resin-bound functional sites, resulting in an increased rate of amide bond production. The peptides yield and purity obtained from new support was high when compared to Merrifield resin.

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