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Solid phase synthesis of nuclear localization signal on a new PS-TTEGDA polymer support

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Tetraethyleneglycol diacrylate (2%) cross-linked polystyrene a highly solvating copolymer is used for the synthesis of the peptide. The peptide is grown from the hydroxymethyl functional site introduced into the resin. The peptide H-Ala-Ala-Ala-Lys-Lys-Asn-Ser-Leu-Ala-Leu-Ser-Leu-Thr-Ala-Asp-Gln-Met-Val-Ser-Ala-Leu-Leu is the sequence of the nuclear localization signal 1 and also the hormone binding domain of the human estrogen receptor (amino acids 302-320) with polyalanine at *N*-terminus. The *C*-terminal amino acid Fmoc-Leu is anchored using DCC and HOBt where the reagents are reacted in 4:4:3 ratio. The remaining amino acids are incorporated into this support following the standard solid-phase methodology of peptide synthesis. The completely deprotected peptide is cleaved from the resin using trifluoroacetic acid in **7h**, isolated in solid form and purity is checked by HPLC. The peptide is characterised by amino acid analysis and MALDI TOF MAS. Biological studies reveal that the peptide is successful in binding three proteins of 73, 55 and 28 kiloDaltons from goat uterine cytosol.

Keywords: PS-TTEGDA polymer support, solid phase synthesis, nuclear localization signal

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Polymer-supported chemical synthesis of peptides is having a wide application in fundamental research and the preparation of bioactive peptides, especially in high value pharmaceuticals[1,2]. Since the solid support plays a key role in SPPS, development of new resins with high mechanical and chemical stability, swellability and compatibility with a wide range of hydrophilic and hydrophobic solvents has been a challenge for polymer chemists ever since the introduction of divinyl benzene cross-linked polystyrene (PS-DVB) resin by Merrifield in 1963 (ref. 3). PS-DVB resin is rigid and its hydrophobic macromolecular network is incompatible with the growing peptide chain[4]. This leads to poor accessibility of the resin bound functional groups to reagents and solvents resulting in the formation of truncated and deletion peptide sequences. In order to overcome the various drawbacks of PS-DVB resin, a series of styrene and non-styrene based supports were developed and have found varying degrees of application in SPPS[5-13]. Tetraethyleneglycol diacrylate cross-linked polystyrene (PS-TTEGDA) was developed as a new support with high mechanical and chemical stability and excellent swelling in various solvents used for SPPS[14]. The hydrophilic polyoxyethylene cross-links imparts a hydrophilic-hydrophobic balance to the resin making it compatible with the growing peptide chain. It was observed that in the case of PS-TTEGDA resin, the *C*-terminal Fmoc-amino acid could be incorporated in **7h** using DCC/HOBT[15] and a completely deprotected peptide can be obtained from the support in **6-7h** at 45-50°C. As a result, the target peptide can be synthesized in a lesser time using this resin compared to the conventional resins.

Proteins, primarily localized in the nuclei, contain a set of amino acid sequences known as nuclear localization signals. They are essential for targeting proteins from the extracellular matrix to the nucleus through the nuclear envelope[16]. Synthesis of nuclear signal sequence is essential to study the mechanism of transport of proteins. In this paper, we report the

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*Address for communication: TC-27/1834, Sri Chithira Lane, Vanchiyoor, Trivandrum 695 035, Kerala ability of 2% PS-TTEGDA resin for the synthesis of the 22-residue peptide which is the sequence of the nuclear localization signal 1 and also the hormone binding domain of the human estrogen[17] receptor (302-320) with polyalanine *N*-terminus. In this synthetic strategy stepwise coupling and deprotection steps were observed to proceed in near quantitative yield supporting the positive role of the hydrophilic and flexible polyoxyethylene cross-links in facilitating the synthetic reactions.

The first amino acid Fmoc-Leu was incorporated to the hydroxymethyl resin using DCC and HOBt. The completely deprotected peptide was separated from the resin in **7h** using anhydrous trifluoroacetic acid (TFA) and the purity of the peptide was checked by HPLC. The crude peptide obtained in 95% yield was characterised by amino acid analysis and MALDI-TOF mass spectrum. This synthesis confirms that PS-TTEGDA resin is much superior to the conventional resins and it would find application as a competent solid support for the synthesis of biologically active polypeptides in high purity and homogeneity.

Results and Discussion

The success of solid phase peptide synthesis depends upon the accessibility of the resin bound functional groups to reagents and solvents. An ideal support should have an optimum hydrophobic/hydrophilic balance so that it can swell uniformly in both polar and non-polar solvents. The support was synthesized by copolymerising two mole percent hydrophilic TTEGDA and hydrophobic styrene by free radical aqueous suspension polymerisation using benzoylperoxide as free radical initiator. The monomers were mixed with the diluent toluene and suspended in water containing 1% polyvinylalcohol (MW~72,000) which served as suspension stabilizer. The polymer was obtained in uniform spherical beads of 200-400 mesh size. The physico-chemical properties of a polymer support are determined by the chemical nature of the monomers and the mole percentage of the cross-linker. The IR spectrum of the powdered resin (KBr) shows intense peak at 1720 corresponding to ester carbonyl and 1150 cm^{-1} corresponding to the ether linkage of the cross-linker besides the usual peaks of polystyrene. Solid state ^{13}C-CP-MAS NMR spectrum of the resin shows an intense peak at 127.89 ppm corresponding to the C-3 carbon of the polystyrene ring. The backbone methylene carbon of the polymer appears as a single peak at 40.34 ppm. The methylene carbon of ether linkage of the cross-linking agent appears as a small peak at 70.65 ppm.

Solvation is a necessary condition favouring organic chemical reactions in gel phase. The two mole percent PS-TTEGDA resin shows higher effective swelling in both polar and non-polar solvents compared to 1% PS-DVB resin (**Figure 1**). In solvents like DCM, DMA, DMF and NMP the polymer swells like a gel form, which is an indication of easy site accessibility and thus resin functionalisation. Functionalisation of this support will not change swelling characteristics of the support. The support was extremely stable under all conditions of peptide synthesis.

Functional sites were introduced in PS-TTEGDA resin by chloromethylation using chloromethyl methyl ether (CMME) in presence of anhydrous $ZnCl_2$ solution in THF[18]. CMME being a good swelling agent for the polymer, chloromethylation proceeds easily. The reaction can be controlled to produce chloromethyl resin of desired chlorine capacity by varying the amount of CMME, catalyst, temperature and duration of reaction. The degree of chloromethylation in the resin was determined by Volhardt's method[19]. The chloromethyl resin was converted to hydroxymethyl resin by heating with potassium acetate followed by hydrazinolysis[20]. The resin shows characteristic IR (KBr) bands at 3424 corresponding to hydroxyl group, 1722, 1490 corresponding to the ester carbonyl and 1150 cm^{-1} corresponding to the ether linkage of the cross-linker. The nature of polymer/peptide interaction and the successful application of various reaction conditions in 2% PS-TTEGDA resin are revealed through the synthesis of the 22 residue nuclear localization signal sequence. The peptide was synthesized using Fmoc-Chemistry. The *C*-terminal Fmoc-Leu was incorporated into the hydroxymethyl resin via an ester bond. The reagents Fmoc-Leu/DCC/HOBt were reacted in the ratio 4:3:3 in DMA for 7 hr at room temperature to get Fmoc-Leucyl resin of capacity 0.32 mmole/g. The peptide was assembled manually using 3 mmole excess (with respect to Fmoc-Leu capacity of the resin) of Fmoc-amino acid, DCC and 3.5 mmole excess of HOBt as their preformed active esters. The coupling reaction was allowed to take place for 50 min in NMP. Single coupling was sufficient for complete incorporation of each residue which was evident from the semi-quantitative ninhydrin test[21]. In the case of Fmoc-Asn(Trt), the 17th residue from *C*-terminus, appreciable percentage

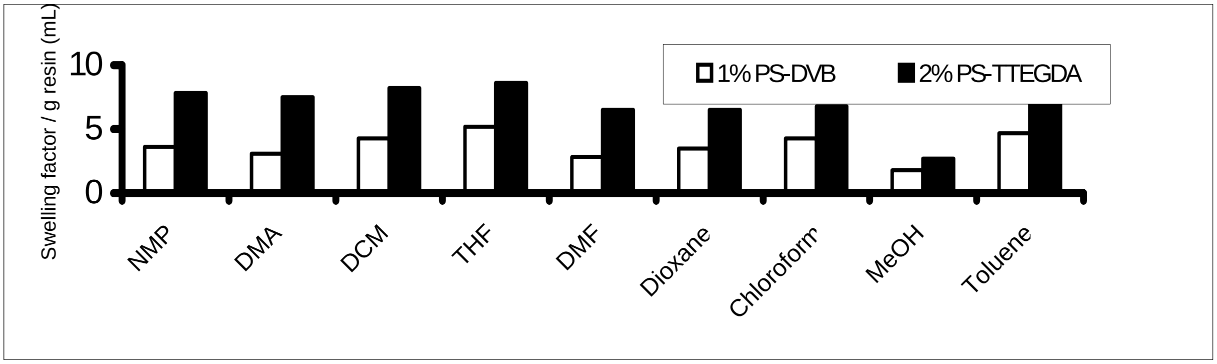


Figure 1—Swelling comparison between 2% PS-TTEGDA resin and 1% PS-DVB resin in various solvents. This was determined by weight gain as solvent /g of dry bead reduced volume of solvent /g dry resin.

of reaction was not observed even after 2 hr of coupling time. The coupling reagent DCC/HOBt was replaced by HBTU and the reaction was carried out using Fmoc-Asn(Trt), HBTU, HOBt and DIEA, for 50 min. The last five amino acid residues were incorporated following HBTU coupling method. HBTU can very effectively drive to completion couplings which are difficult due to internal aggregation via β-pleated sheet formation or by association between peptide and support matrix. HOBt acts as a catalyst and the base DIEA enhances the reaction by increasing the *p*H of the medium. Different steps involved in the synthesis are shown in **Scheme I.**

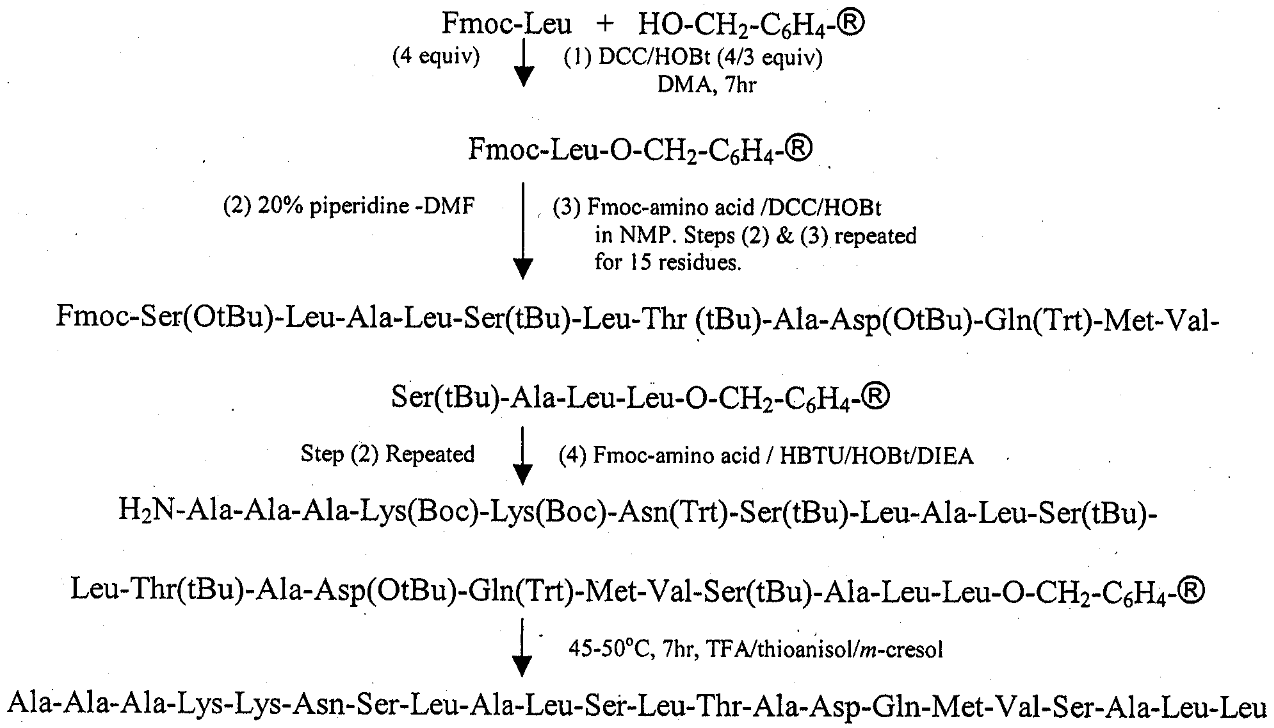
The yield of the peptidyl resin from 100 mg of the starting PS-TTEGDA resin was 170 mg. The peptide was cleaved from the support by suspending the peptide resin in trifluoroacetic acid, and thioanisol, *m*-cresol scavengers for 7 hr at temperature 45-50°C. The crude peptide was obtained in 95% yield. Purity of the peptide was checked by HPLC where a linear gradient of buffers 0.1%TFA in water (A) and 0.1%TFA in acetonitrile (B) was used (**Figure 2**). HPLC showed a single major peak indicating the homogeneity of the peptide synthesized. The enhanced reactivity and purity of the peptide highlights the positive role of the cross-linker, which provides a hydrophilic/hydrophobic balance and greater chain mobility to the support, which helps to meet the requirements for ideal polymeric support. Anchoring of Fmoc-amino acids using DCC/HOBt and cleavage at 45-50°C is well established in 2% PS-TTEGDA resin through the synthesis of this peptide. Cleavage at this temperature facilitated the separation of the peptide from the support in lesser time without the use of a linker.

Amino acid analysis of the peptide confirmed the sequence Leu, 5.02(5); Ala, 6.02(6); Ser 2.22(3); Val 1.04(1); Met 0.95(1); Glu 0.99(1); Asp 1.94(2); Lys 1.98(2). The MALDI-TOF MS result infers that the major peak obtained in analytical HPLC belong to the target peptide. m/z 2217.71 $(M + H)^+$, $C_{96}H_{170}O_{31}N_{26}S_1$ requires M^+ 2216.68.

Biological activity of the peptide was studied[22] by coupling the peptide to cyanogen bromide activated sepharose 4B and column chromatographing goat uterine cytosol over the peptide sepharose. The peptide bound proteins were eluted and subjected to sodiumdodecyl sulphate-polyacrylamide gel electrophoresis. The proteins of molecular mass 73,55 and 28 kiloDaltons were found to bound to the column revealing the binding and transporting ability of the synthesized peptide.

Experimental Section

Materials: Styrene, TTEGDA, Polyvinyl alcohol (PVA, MW ~ 72,000) were purchased from Aldrich Chemical Co., USA. Dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt), 2-(1-*H*-benzotriazol-1-y1) 1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU), piperidine, Fmoc-amino acids were purchased from Novabiochem Ltd., UK. Diisopropylethylamine (DIEA) and thioanisol were purchased from Sigma Chemicals Company, (USA). Chloromethyl methyl ether (CMME) was prepared using the literature procedure. Solvents used were of HPLC grade purchased from E. Merk (India) and SRL (India). IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The ^{13}C CP-MAS solid-state NMR measurements were conducted on a Brucker 300 MSL CP-MAS instrument operating at 75.47 MHz. HPLC was done on a Pharmacia Akta purifier using C-18 reverse



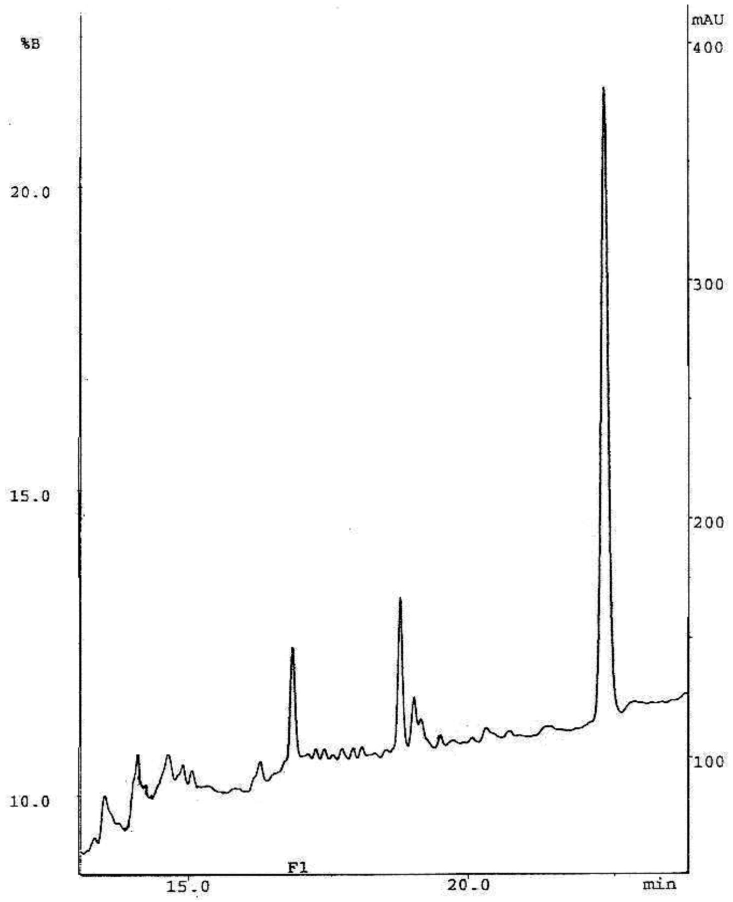
Scheme I—Synthesis of 22 residue nuclear localization signal sequence on 4-hydroxymethyl 2% PS-TTEGDA resin[2]. ®- PS-TTEGDA resin

phase semi preparative HPLC column. The amino acid analysis was carried out on an LKB 4151 Alpha plus amino acid analyzer. Mass spectra of peptide were recorded in a Kratos PC-Kompact MALDI-TOF mass spectrometer.

PS-TTEGDA Support. Styrene and TTEGDA were washed free of the inhibitors using 1% NaOH solution followed by distilled water and drying over anhydrous calcium chloride. A net volume of 1% PVA (MW ~ 72,000, 8 times the volume of monomers and diluent) was prepared in double distilled water and kept at 1600 rpm at 80°C under a slow stream of nitrogen in the reaction vessel. A mixture of styrene (30.62 g), tetraethylene glycol diacrylate (1.8 g) and recrystallized benzoyl peroxide 600 mg were mixed with toluene (12 mL) the inert diluent and added to the PVA solution. The reaction was continued for 6 hr and the copolymer was obtained as shining beads. The polymer was washed free of the stabilizer and the unreacted monomers by treating with distilled water, acetone and methanol. Finally, the polymer was further purified by soxhlet extraction with DCM, acetone and dried under vacuum. Yield: 25 g of dry resin.

Chloromethyl PS-TTEGDA resin. 2% PS-TTEGDA resin (2 g) was allowed to swell in DCM for 1hr and the excess DCM was decanted. A mixture of chloromethyl methyl ether (12 mL) and 1M $ZnCl_2$ in THF (0.4 mL) was added to the swollen resin and was refluxed at 40°C for 3.5 hr. The resin was filtered using a sintered glass funnel and washed with THF (3 × 20 mL), THF/water (1:1, 3 × 20 mL), THF/3 *N* HCl (1:1, 3 × 20 mL), THF (3 × 20 mL) and methanol (3 × 20 mL) and dried in vacuum. The chlorine capacity of the resin was 0.38 mmole/g as estimated by Volhardt's method.

Hydroxymethyl PS-TTEGDA resin. Chloromethyl PS-TTEGDA resin (0.38 mmole Cl/g, 1g) was stirred with potassium acetate (0.36 g, 3.8 mmole) in methylcellosolve (6 mL) at 125-135°C. After 24 hr, the acetoxy resin was filtered, washed with DCM (3 × 25 mL) and NMP (3 × 25 mL). The acetoxy resin was suspended in DMF (6 mL) containing hydrazine hydrate (178 µL, 3.8 mmole) for 72 hr. The resin was filtered, washed with DMF (3 × 25 mL), methanol (3 × 25 mL), DCM (3 × 25 mL), ethanol (3 × 5 mL) and vacuum dried. From this 100 mg resin was acetylated with acetic anhydride-piperidine mixture (1:4, 3 mL). After 6 hr the above mixture was refluxed with distilled water (10 mL) for 3 hr, cooled and filtered. The acetic acid formed was back titrated with 0.1*N* NaOH. Resin capacity = 0.34 mmole/g.



dry resin.

Figure 2—HPLC of crude nuclear signal sequence on PS-TTEGDA resin using a linear gradient of buffers 0.1% TFA in water (A) and 0.1% TFA in acetonitri[le (B). Flow rate 0.5mL/min

Synthesis of nuclear signal sequence

Anchoring of *C*-terminal Fmoc-Leu. Fmoc-Leu (48 mg, 0.14 mmole, 4 equiv) was dissolved in 0.2 mL DMA and mixed with 14 mg of HOBt (0.1mmole, 3 equiv) in 0.1mL DMA. The mixture was chilled in an icebath and a cold solution of DCC (28 mg, 0.14 mmole, 4 equiv) in DMA was added. The resulting solution was stirred cold for 5-10 min and then added to 100 mg (0.34 mmole OH/g) dry resin. The mixture was stirred occasionally at room temperature for 7 hr. Then, the resin was washed with CH_2Cl_2 × 3, isopropanol × 3, CH_2Cl_2 × 3, isopropanol × 3, acetic acid × 2, isopropanol × 3, CH_3OH × 3 and dried in vacuum. The degree of incorporation of Fmoc-Leu was estimated from the O.D. measurements at 290 nm, 0.32 mmole/g.

Stepwise incorporation of amino acid residue. Peptide was synthesized manually in a silanized glass reaction vessel of 15 mL volume with a sintered filter at one end. The target peptide was assembled on Fmoc-Leu resin using the respective Fmoc-amino acids. Fmoc-amino acids, Fmoc-Ser(tBu), Fmoc-Gln (Trt), Fmoc-Asp(OtBu), Fmoc-Thr(tBu), Fmoc-Asn(Trt), Fmoc-Lys(Boc) were the side chain protected amino acids used for synthesis. For each acylation cycle preformed active esters of 3 fold molar excess of Fmoc-amino acid, DCC and 3.5 molar excess HOBt were used to drive the reaction to completion. Single coupling of 50 min in NMP was sufficient for the complete incorporation of each residue. The extent of substitution was monitored by semi-quantitative ninhydrin test. The 17th residue Fmoc-Asn(Trt) did not show appreciable percentage of reaction on coupling using the active ester of DCC/HOBt. A second coupling was performed using the coupling reagent HBTU/DIEA where Fmoc-Asn(Trt) (3 equiv) mixed with HOBt (3 equiv), HBTU (2.98 equiv) and DIEA (3.5 equiv) dissolved in minimum amount of NMP and added to the Fmoc-deprotected peptide resin. Coupling reaction was complete in 50 min. The remaining five amino acid residues were incorporated following the same method. After the incorporation of all the amino acid residues, Fmoc protection of target peptidyl-resin was removed and the resin was washed with DMF (5 mL × 6 × 1 min), CH_2Cl_2 (5 mL × 6 × 1 min) MeOH (5 mL × 4 × 1 min) and dried under vacuum. Yield of peptide resin = 170 mg.

Removal of peptide from the polymer support. The nuclear signal sequence was cleaved from the peptide resin by suspending 100 mg of the peptidyl resin in a mixture of TFA (3 mL), thioanisol (0.3 mL) and *m*-cresol (0.3 mL) at 45-50°C. After 7 hr, the suspension was filtered, washed with TFA (1 mL), DCM (5 mL) and the filtrate was concentrated under reduced pressure at 40°C until a thick oily residue was obtained. To the filtrate ice-cold diethyl ether (10 mL) was added, and the precipitated peptide was washed with ice-cold ether (6 × 10 mL) to remove the scavengers and dried. The cleavage yield was 95% as per the weight of the crude peptide. Purity of the peptide was checked on a C-18 reverse phase HPLC column using a linear gradient of solvent, 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The HPLC profile of the peptide showed only one major peak corresponding to the target peptide which was confirmed by amino acid analysis and MALDI-TOF MS.

Binding property of the nuclear localization signal. The peptide synthesized was dissolved in 0.2 M $NaHCO_3$ buffer of *p*H 9.3 and transferred to a 10 mL column of cyanogen bromide activated sepharose 4B. One milligram of the synthetic peptide was coupled per 1 mL of the packed sepharose. The column was washed and equilibrated with the TEMN buffer which is 50 m*M* Tris-HCl, *p*H 7.6 containing 1m*M* ethylene-diamine tetracetic acid, 12 m*M* monothioglycerol, 0.2 m*M* phenyl methyl sulphonyl fluoride and 50 m*M* NaCl. Goat uterine cytosol homogenized in TEMN buffer was chromatographed over the peptide-sepharose column and the flow-through fractions from the column were recycled for maximal binding. The column was washed extensively with the TEMN buffer and the peptide bound proteins were eluted using a linear gradient of NaCl (0-1*M*) in TEMN buffer. The fractions were collected and their absorbance was measured at 280 nm. The eluted proteins appeared as a single peak of absorbance at 280 nm. These fractions were concentrated and subjected to sodiumdodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). The silver stained gel displayed three proteins of molecular mass 73, 55 and 28 kiloDaltons, ensuring the binding and transporting ability of the synthesized peptide.

Conclusion

In summary, a new PS-TTEGDA resin was developed by aqueous suspension polymerisation of the respective monomers. A 2% PS-TTEGDA resin showed a hydrophobic-hydrophilic balance, good mechanical stability, excellent swelling properties and performance in the stepwise synthesis of the peptide. Efficiency of the support is demonstrated by synthesizing the 22 residued nuclear localization signal sequence which is used to study the mechanism of binding and transporting of proteins. The *C*-terminal Fmoc-amino acid incorporation using DCC/HOBt and cleavage at 45-50°C is well established on PS-TTEGDA resin through this synthesis. The high yield, purity and biological activity of the peptide synthesized confirm the efficiency of the support in polypeptide synthesis.

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