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Gel-Phase Peptide Synthesis on a New High-Capacity Tetraethyleneglycol Diacrylate-Crosslinked Polystyrene Support: Synthesis of Pardaxin 16-33.

M. RENIL,[a] R. NAGARAJ[b] AND V.N. RAJASEKHARAN PILLAI[a]*

[a]School of Chemical Science, Mahatma Gandhi University, Kottayam 686 560, India.

and

[b]Centre for Cellular and Molecular Biology, Hyderabad 500 007, India.

Abstract: An insoluble but highly solvating copolymer composed of tetraethyleneglycol diacrylate (TTEGDA)-crosslinked polystyrene has been prepared in beaded form by free-radical suspension polymerization of the monomers and employed as a new solid support for gel-phase peptide synthesis. This new polymer support has comparable physical and mechanical properties as that of the divinylbenzene (DVB)-crosslinked polystyrene support permitting identical manipulation such as shaking and filtration when used as a support for solid-phase peptide synthesis. This polystyrene-TTEGDA crosslinked polystyrene resin undergoes much more effective swelling and solvation than DVB-crosslinked polystyrene in a range of solvents with widely varying polarity which are commonly employed in peptide synthesis. The resin can be easily chloromethylated under controlled conditions to prepare resins of varying capacity ranging from low (0.15 meq/g) to high capacity (3.5 meq/g). A high capacity resin with 1.5 meq/g chlorine capacity was used for synthesis of hydrophilic C-terminal 18-residue peptide of pardaxin from *Pardachirus Pavoninus*. The peptide was cleaved from the polymeric support by trifluoroacetic acid at 37°C and purified by Fast Protein Liquid Chromatography (FPLC). The purified peptide was shown to be homogeneous by HPLC and the identity of the 18-residue peptide was confirmed by amino acid analysis. The free peptide possesses a disordered conformation as revealed by the CD measurement.

INTRODUCTION

Polystyrene crosslinked with divinylbenzene(DVB) has been widely used in solid phase peptide synthesis with considerable success.[2-4] However extreme purity and homogeneity of medium to large peptide is still a challenging problem in peptide synthesis using this support.[5,6] This is mainly due to the rigid and hydrophobic macromolecular environment created by the DVB-crosslinked polystyrene matrix. A number of approaches have been suggested to overcome the difficulties associated with this type of resins.[7,8] In order to increase the polarity of the support and make it compatible with the growing peptide chain polar polyacrylamide type supports were developed and have been successfully employed in peptide synthesis.[9,10] They show effective swelling in polar solvents but their swelling property in non-polar solvents is rather poor. Their mechanical stability is also less compared to the polystyrene resins. For solid phase peptide synthesis the support must swell effectively in both polar and non-polar solvents. For this the polymer support should have an optimum hydrophobic-hydrophilic balance. In order to achieve this balance in a single macromolecular matrix we deemed it of interest to copolymerise the hydrophobic styrene and hydrophilic flexible tetraethyleneglycol diacrylate (TTEGDA) and to employ the resulting copolymer as a carrier for peptide synthesis. In this new copolymer the functional group reactivity is very much enhanced due to the greater chain mobility of the flexible oligooxyethylene crosslinking when compared to the rigid hydrophobic DVB-polystyrene resin. It was observed that even high-capacity chloromethylated resin could be used effectively for the solid phase synthesis in the case of this new resin.

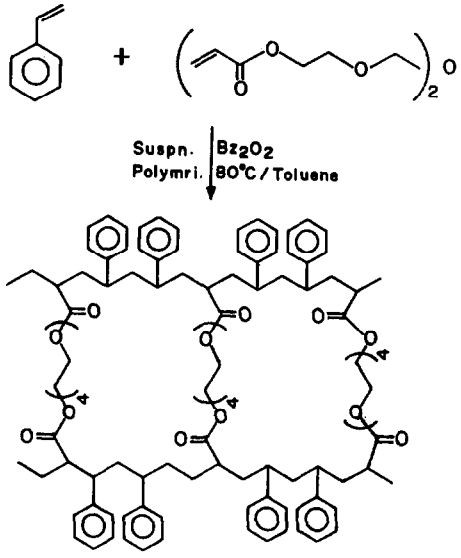
We report herein the use of a high capacity chloromethylated 2% TTEGDA-crosslinked polystyrene support for the synthesis of a 18-residue peptide corresponding to the C-terminal region of pardaxin, a shark repellent peptide toxin isolated from the secretion of the Red Sea Moses Sole fish.[11,12] Because of the highly

flexible and hydrophilic crosslinking the support can accommodate the growing peptide chain even at high capacity. The stepwise coupling and deprotection steps in this synthetic strategy were observed to proceed in near-quantitative yield supporting the positive role of the hydrophilic and flexible polyoxyethylene crosslinks in facilitating the synthetic reactions. This new crosslinked copolymer could be regarded as an ideal solid support with balanced hydrophobic-hydrophilic characterstics which are favourable for solid phase peptide synthesis.

RESULTS AND DISCUSSION

Suspension polymerization has been proved to be the most useful technique for synthesising crosslnked polymeric support, principally because of the extremely convenient physical form of the beaded product which lends itself to further conversions. The chemical nature and the topographical structure of the polymer matrix are the two important factors which determine the physicochemical properties that render a polymer support favourable for peptide synthesis. The topography of the polymer matrix is determined by the chemical nature of monomers and the mole percentage of crosslinking agent. The crosslinking provides the desired mechanical integrity for the resin. Polystyrene crosslinked with 2 mole % TTEGDA was prepared by the free-radical aqueous suspension polymerization[13] and used as a carrier for solid phase peptide synthesis of the 18-residue peptide (**Scheme 1**). The insoluble support was obtained as spherical uniform beads as observed in the scanning elctron micrographs (not shown). We could get reproducible results in the preparation of beads of 100-500 mesh size by adjusting the amount of stabilizer polyvinyl alcohol, geometry of the vessel and stirrer, and the stirring rate.

These polymer beads were characterised by infrared spectroscopy and ^{13}C CP-MAS NMR spectroscoy. IR spectrum shows a sharp band at 1720 cm-1 corresponding to the ester carbonyl and a broad band at 1150 cm-1 characteristic of ether linkages of the crosslinking agent (**Fig.1a**). Solid state ^{13}C CP-MAS NMR spectrum (**Fig.1b**) shows an intense peak at 127.89 ppm which corresponds to aromatic polystyrene carbons and a small peak at 145.65 ppm arising from C-3 carbon of the styrene. The backbone methylene carbon of the polymer



Scheme 1. Preparation of TTEGDA-crosslinked polystyrene support.

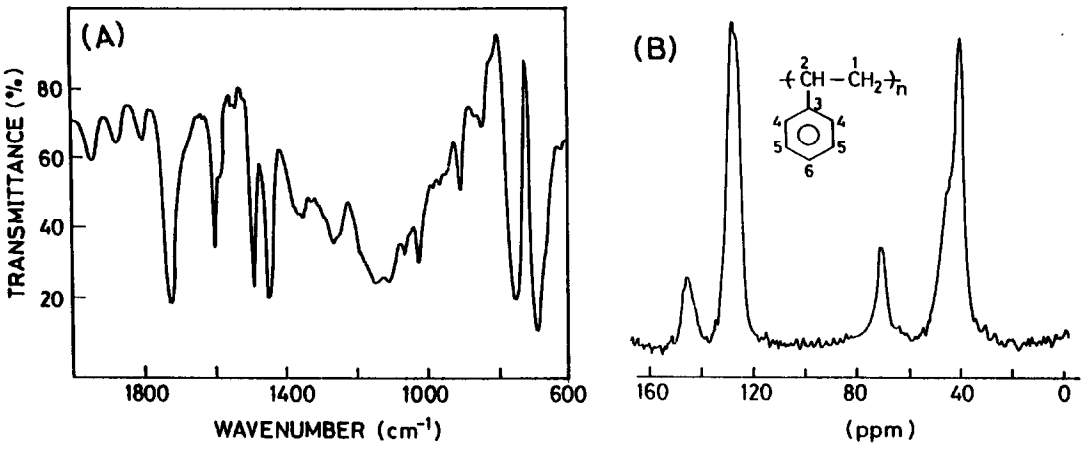


Fig.(1a) IR (KBr) Spectrum and **(1b)** ^{13}C-CP-MAS NMR(solid state) spectrum of TTEGDA-crosslinked polystyrene resin.

Boc-Glu(OBzl)-O-RESIN

1) 30% TFA/CH_2Cl_2
2) Neutralization DIEA-CH_2Cl_2
3) Boc-Gln, DCC, HOBt (1:1:1)
4) Repetition of steps 1-3 with successive amino acid units.

Boc-Lys(Cl-Cbz)-Thr-(Bzl)-Leu-Leu-Ser(Bzl)-Ala-Val-Gly-Ser(Bzl)-Ala-Leu-Ser(Bzl)Ser(Bzl)-Ser(Bzl)-Gly-Glu(OBzl)-Gln-Glu(OBzl)-O-RESIN

TFA:Thioanisole:m-cresol (10:1:1), 37°C

H-Lys-Thr-Leu-Leu-Ser-Ala-Val-Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Glu-Gln-Glu-OH

Scheme 2. Outline of the synthesis of 18-residue peptide using TTEGDA-crosslinked polystyrene resin as the solid support.

appears as a single at 40.38 ppm. The methylene carbon of ether linkages of the crosslinking agent TTEGDA appears as a small peak at 70.65 ppm.

The resin was functionalized for the peptide synthesis by chloromethylation using chloromethylmethyl ether and $ZnCl^2$/THF as catalyst[14]. Chloromethyl resin of high capacity 1.5 meq Cl/g was used for the present synthesis. The use of high-capacity poly[N-[2-(4-hydroxyphenyl)ethyl]-acrylamide] support have been reported for solid phase peptide synthesis with great success[15]. But not much success was seen in the case of high-capacity DVB-crosslinked polystyrene support[16]. Boc-Glu(OBzl) was first attached to the chloromethyl

TTEGDA-crosslinked polystyrene resin by the cesium salt method.[17] Boc-Glu(OBzl) was quantatively attached to the solid support. The extent of incorporation of amino acid was estimated by amino acid analysis and picric acid titration method.[18] The reaction was also confirmed by the absence of any detectable amount of residual chlorine by Volhardt's method.[19] Standard procedure for the solid phase method with certain modification as presented in **Scheme 2** was followed for the synthesis of the 18-residue peptide.[16] The reaction flask with the Boc-Glu(OBzl)-polymer was placed in a shaking vessel and the remaining 17 amino acid residues were incorporated in the manual mode. Upto 3 residues could be incorporated per day using this manual procedure. N-Boc protection was used throughout the synthesis along with suitable selction of side chain protecting groups as detailed in the experimental section. After Boc-deprotection using 30% TFA in CH_2Cl_2 and neutralization with 5% diisopropylethylamine (DIEA) in CH_2Cl_2, 2.5 eq. Boc-amino acid was added to the resin followed by treating with 2.5 eq. of DCC in CH_2Cl_2 for 45 min. HOBt procedure was used for Boc-Gln-coupling. Double coupling was followed throughout the synthesis. Completion of the coupling was verified by the ninhydrin test.[20] A third coupling was performed wherever necessary to bring the reaction up to 99.8% completion. The finished peptide was cleaved from the resin by treating the peptide resin with TFA in the presence of thioanisole and m-cresol at 37-40°C for 22 hr. under perfectly anhydrous conditions.[21,22] A second cleavage was carried out to make sure that complete cleavage of the peptide from the support has occured. The cleavage yield was 98% as indicated by the estimation of the remaining peptide bound to the resin. The cleaved peptide was separated by filtration and subsequent removal of TFA by rotary evoporation. The peptide was precipitated using cold ether and it was washed with ether to remove the scavenging reagent. The completely deprotected peptide was purified on a C-18 reverse phase semi prep Pharmacia column on a FPLC. The solvent system used was 0.25% TFA in water (A) and 0.25% TFA in acetonitrile (B) at a flow rate of 0.5mL per min. The gradient used is shown in **Fig.2**, a single major peak which occured at 38% acetonitrile was collected and the

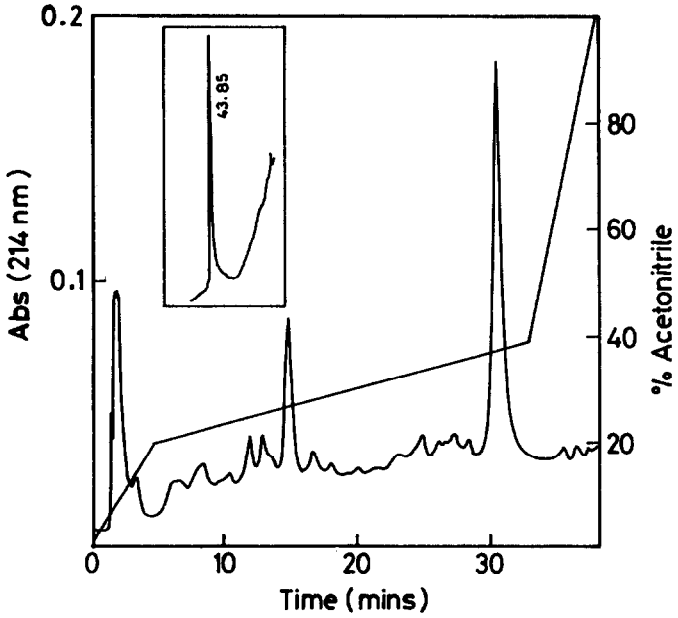


Fig.2: FPLC of unpurified 18-residue Paradxin sequence after TFA cleavage. Gradient used 0 time 0% B; 20% B in 5 min; 39% B in 30 min; 100% B in 5 min ; a flow rate: 0.5 mL/min.(Inset): HPLC profile of the purified peptide. Gradient used: 0 time 5% B; 40% B in 35 min; 40% for 5 min; 95% B in 25 min; flow rate: 0.8 mL/min.

solvent evaporated to get 67.5% overall yield of the pure peptide. Amino acid analysis of this fraction agrees with the target sequence. From the analytical HPLC profile of the purified peptide (**Fig.2**) the purity of the 18-residue peptide was confirmed. The free peptide in methanol showed a circular dichrosim curve with a weak positive peak at 215 nm and strong negative band near 197 nm suggesting a random coil structure.

Table 1: Comparison of swelling characteristics of crosslinked Polystyrene gels

Solvent	Swelling Factor*	
	1% DVB-PS Resin mL	2% TTEDA-PS Resin ml
Chloroform	4.3	6.8
Tetrahydrofuran	5.2	8.1
Toluene	4.7	7.5
Pyridine	4.2	6.5
Dioxane	3.5	6.4
Dichloromethane	4.3	8.5
DMF	2.8	6.5
Methanol	1.8	2.8

*** Determined by gain in weight as solvent/g of dry beads reduced volume of solvent/g dry beads.**

The present investigation clearly demonstrates the utility of the newly developed TTEGDA-crosslinked polystyrene polymer as a carrier for stepwise synthesis of peptides. This copolymer has the desired physico-chemical properties such as optimum hydrophilic-hydrophobic balance and greater chain mobility. The resin can be subjected to easy operation such as washing with different solvents and simple filtration. Another superior property of this copolymer becomes apparent from the preliminary kinetic studies which indicated that coupling of *p*-nitrophenyl acetate with the aminomethyl TTEGFDA-crosslinked polystyrene beads proceeds at a much faster rate compared to the aminomethyl DVB-crosslinked polystyrene resin.[23] TTEGDA-crosslinked polystyrene resin shows mechanical stability characteristics similar to DVB-crosslinked polystyrene. However the TTEGDA-crosslinked polystyrene resin swells in most of the organic solvents to a greater extent than the DVB-crosslinked polystyrene resin (**Table 1**). These supports are extremely stable under all conditions of peptide synthesis. Compared to the classical solid phase synthesis at conventional loading these high capacity resins have a number of advantages which stem from the much more efficient use of available volume within each gel particle. These advantages include enhanced coupling rate during peptide bond formation, major savings in cost due to the more effective use of reagents and washing solvents, greatly improved sensitivity on monitoring of the coupling reaction to effect peptide bond formation and the synthesis of peptide in a relatively large scale.

EXPERIMENTAL

General. Styrene, TFA and TTEGDA were obtained from Aldrich chemical company USA and thioanisole from Fluka. Boc-Glu(OBzl), Boc-Thr(Bzl) and Boc-Lys(ClCBz) were obtained from Peninsula Labratories USA. DCC and DIEA were purchased from Sigma chemical company USA. Other Boc-amino acids were prepared according to Schnabel's procedure.[22] Chloromethylmethyl ether (CMME) was prepared using literature procedure.[23] IR spectra were recorded on a Shimadzu IR 470 spectrometer in KBr pellets. The ^{13}C-CP-MAS solid state NMR measurements were conducted on a Bruker 300 MSL CP-MAS instrument operating at 75.47 MHz. The spectra were run with fine powders of polymer beads at room temperature and the Kelf rotor was employed for MAS. The samples were rotated with a spectral width of 25,000 Hz, the CP

time was 22 ms and the number of scans was in the range of 200-300. Each sample was rotated with two different spin rates and by comparing the resultant spectra the spinning side bands were eliminated. For HPLC analysis a Shimadzu Model 6 A liquid Chromatograph equipped with a SPD 6 A uv spectrophotometric detector and a C-R6A chromatopac electronic plotter was employed. FPLC was done on a Pharmacia instrument on a C-18 reverse phase semi prep FPLC column. The amino acid analysis was carried out on a LKB 4151 Alpha plus amino acid analyzer. For this the peptide was hydrolysed using 6N HCl-TFA (2:1 ratio) in a pyrex glass tube fused under nitrogen for 15 hr at 130°C. CD spectrum was recorded on a Jasco J 500A spectropolarimeter attached with a Jasco DP-501 N data processor.

TTEGDA-crosslinked polystyrene copolymer. In a typical experiment a four-necked reaction vessel equipped with a thermostat, teflon stirrer, water condenser and nitrogen inlet was used. Polyvinyl alcohol (0.5 g) dissolved in double distilled water (200 mL), calcium suplhate (5 mg) and calcium phosphate (10 mg) were added to the vessel. A mixture of styrene (25.5 g) tetraethyleneglycol diacrylate (1.5 g) and benzoyl peroxide (0.5 g) dissolved in benzene (20 mL) was added to the vessel by stirring the aqueous solution at 400 rpm. The temperature was maintained at 80-C using a themostated water bath. The entire reaction was carried out under a slow stream of nitrogen. After 20 hr the solvent-embedded copolymer beads were washed free of stabilizer and unreacted monomers by treating with distilled water, acetone, chloroform and methanol. The copolymer was further purified by refluxing with trifluoroacetic acid to remove any impurity for 6 hr. The polymer beads were filtered, washed with CH_2Cl_2 and CH_3OH, dried under vacuum at 40°C for 10 hr to yield 25 g. of dry beads (92.5%).

Chloromethyl TTEGDA-crosslinked polystyrene resin. A mixture of 2% TTEGDA-crosslinked polystyrene resin (5 g), chloromethylmethyl ether(30 mL) and CH_2Cl_2 (25 mL) was stirred for 5 min followed by addition of 1.0 M $ZnCl_2$ in THF (2 mL). The suspension was refluxed at 45-50°C for 7 hr. The resin was filtered and washed with CH_2Cl_2, THF/H_2O, THF/3 N HCl, THF and CH_3OH. Drying under vacuum at 40°C for 10 hr yielded 5.4 g. of the chloromethyl resin. The resin was found to have a capacity of 1.5 mmol Cl/g as estimated by Volhardt's method.

Cesium Boc(OBzl) Glutamate. Boc-Glu(OBzl) (0.33 g, 1 mmol) was dissolved in 4:1 ethanol/water (7 mL) and a 1 M solution of Cs_2CO_3 was added dropwise until the pH was 7.0. The solvent was removed by azeotropic distillation with benzene and the resulting white solid was kept overnight over P_2O_5 under vacuum.

Boc-Glu(OBzl)resin. Boc-Glu(OBzl)-O-Cs from the above step was dissolved in DMF (7 mL) and cholromethyl resin (0.3 g, 0.45 mmol) was added. The mixture was kept at 40-50°C for 22 hr with occasional shaking. The resin was washed with DMF (3x1 min), DMF H_2O (9:1) (5x2 min), DMF/H_2O (4:6) (5x2 min), DMF (3x1 min) CH_2Cl_2 (3x2 min) and finally with CH_3OH. The product resin was dried under vacuum for 8 hr (0.55 g). Amino acid analysis of the resin hydrolysate gave 1.25 mmol of Glu/g of resin.

Peptide synthesis. Boc-Glu(OBzl)-resin (0.2 g, 0.21 mmol) was placed in a solid phase shaking vessel and was then treated stepwise in the manual mode. The ninhydrin test was performed for the detection of incomplete coupling reactions. After the completion of the 17 deprotection and coupling cycles the resin was taken out of the reaction vessel, washed thoroughly with CH_2Cl_2, CH_3OH:$CHCl_3$ (1:1), CH_3OH and dried under vacuum to yield 0.55 g of Peptide resin. Cleavage of the 18-residue peptide from the resin: 100 mg of the peptide resin was suspended in dry TFA (2 mL) and thioanisole (0.2 mL) m-cresol (0.2 mL) were added to this. The mixture was deaerated with nitrogen and kept at 37°C for 22 hr. The polymeric material was filtered off and washed with fresh TFA. The filtrate was evaporated to obtain a thick oily residue which was precipitated as a white powder (56 mg) by the addition of cold ether. The peptide was washed thoroughly with cold ether (5 mL x 8) to remove all the scavenging reagents. The resin was kept for a second cleavage under the same conditions as above to yield an additional crop of 5 mg of the peptide. The peptide was dissolved in methanol and again reprecipitated with cold ether to yield 58 mg of the crude peptide.

Purification of the peptide. The crude peptide (10 mg) in methanol (0.5 mL) was loaded in small portions onto a Pharmacia RP-C-18 FPLC column and eluted with a linear gradient as shown in Fig.3. Solvent(A) is H_2O/0.25% TFA and B is MeCN/0.25% TFA. A flow rate of 0.5 mL/min was maintained. The major peak corresponding to 37% acetonitrile was collected and evoporated to get 7 mg pure peptide. The concentration and purity of the peptide was determined by amino acid analysis. The pure peptide was reinjected onto a Shimadzu RP C-18 analytical HPLC column and eluted with a linear gradient starting from 5% to 40% B in 40 min followed by 5 min eluting with 40% B, where (A) is H_2O/0.1% TFA and (B) 60% MeCN in H_2O/0.1% TFA at a flow rate 0.8 mL/min. The retention time of the peptide under these conditions is 43.85 min (**Fig.2**). Amino acid analysis: Thr 0.60(1); Ser 4.16(5); Glu 3.12(3); Gly 2.02(2); Ala 2.01(2); Val 0.99(1); Leu 3.01(3); Lys 1.02(1). The values for Ser and Thr were found to be somewhat low due to some degradation during hydrolysis.

Swellilng studies of TTEGDA-crosslinked polystyrene. Solvent imbition of the various resins was determined by centrifuge method. A sample of the resin (1 g) was placed in a glass sintered stick (porosity 3) and the latter immersed in the solvent for 1 hr. The stick was then transferred to a centrifuge tube where it was held in position. Excess solvent was removed by centrifuging for 15 min. The stick and the contents were then weighed. Immersion in the solvent was continued for 5 minutes. These operations were repeated until a constant weight increase was achieved. Finally a similar blank experiment was performed using the empty sintered stick. The data was expressed as the volume of solvent absorbed by unit weight of dry resin (mL/g).

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REFERENCES AND NOTES

1. Nomenclature follows the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature, *Biochem. J.* **1984**, *219*, 345. *Eur. J. Biochem.* **1984**, *138*, 9. Abbreviations used: Boc, *tert* butyloxycarbonyl; CBz, benzoxycarbonyl; Bzl, benzyl; DCC, dicychlohexylcarbodiimide; DIEA, N,N-diisopropylethylamine; DVB, divinylbenzene; HOBt, Hydroxybenzatrizole; TFA, trifluoroacetic acid; TTEGDA, tetraethyleneglycol diacrylate.
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