Synthesis and optimization of tri(propylene glycol) glycerolate diacrylate cross-linked polystyrene resin in polypeptide synthesis: role of the macromolecular support in solid phase peptide synthesis

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A novel tri(propylene glycol) glycerolate diacrylate cross-linked polystyrene support for solid phase peptide synthesis was prepared by aqueous radical suspension polymerization. The peptides were grown from the hydroxy functionality of the cross-linker in the polymer and this makes it unique among other styrene-based polymer supports that are currently used in polypeptide synthesis. The role of the polymer support in peptide synthesis was established by studies delineating the optimization of synthetic steps involved in solid phase synthesis. The optimization studies include *C*-terminal amino acid incorporation, N^{α}-Fmoc and Boc-deprotection, acylation reactions and the removal of the target peptide from the support. The dependence between the nature and extent of cross-linking of the polymer backbone and the reactivity of the attached amino groups was investigated by carrying out a reactivity study on amide bond formation compared with Merrifield resin. The resin-like behavior of the new support was studied by synthesizing a 'difficult' sequence of the (34–42) fragment of β-amyloid peptide (1–42) and compared with commercially available Merrifield and Sheppard resins. The synthetic utility of the support was established by synthesizing a 23-residue NR 2B peptide substrate of Ca^{2+}/calmodulin binding peptide in high yield and purity. Better solvation of the resin beads, enhanced coupling efficiency in the peptide synthetic steps and the high yield and purity of the peptides synthesized highlights the positive role of the cross-linker in the new polystyrene support.

Introduction

Since the introduction of solid phase peptide synthesis (SPPS) by R. B. Merrifield, the development of a polymeric support that facilitates the different types of reaction used in polypeptide synthesis has been a challenge to peptide and polymer chemists.[1] Studies on the quantitative aspects of polymer-supported reactions have changed the earlier thinking that the support is an inert or passive medium and have shown that the reactivity of the functional groups attached to a polymeric backbone is governed by various characteristic structural parameters of the polymer support, such as its polarity, the nature and extent of cross-linking, and the solvation of the support and the resin-bound species in the solvent medium.[2] Though Merrifield support has been widely used in SPPS with considerable success, the extreme purity and homogeneity of medium-to-large sized peptides is still a challenging problem to peptide chemists. The physicochemical incompatibility of this support with bound peptides in a solvent medium causes a negative influence on the mass transport of reagents, effective solvation of the polymer as well as the peptide chain, and the coupling and deprotection rates. Many attempts have been made to overcome the difficulties encountered in SPPS especially in the modification of the polymer support. A series of cross-linked polystyrene-based supports with TTEGDA, HDODA and BDODMA cross-linking has been developed and used for SPPS in our laboratory.[3–5] Other types of support developed and tested over these years for SPPS include polyamides, polyethylene glycol–polystyrene graft resins, PEGA, CLEAR, POEPOP, POEPS, CLPSER, and SPOCC resin.[6–13]

In an effort to minimize the physico-chemical incompatibilities of the hydrophobic macromolecular environment of the polystyrene support with the growing peptide chain, a novel support was designed by incorporating tri(propylene glycol) glycerolate diacrylate as a cross-linking agent into the polystyrene core (PS-TRPGGDA). The justification for the selection of TRPGGDA as the cross-linker is the presence of sufficient secondary hydroxy functional sites that can be used as a growth sites for peptide synthesis. Moreover the tri(propylene glycol) chain together with ester functionalities and hydroxy groups confer hydrophilic character on the resultant polymers. An alternative PEG chain can also impart hydrophilicity to the polymer support. The present method of using the cross-linker sites themselves as attaching points for peptide growth is operationally much simpler and hitherto unreported. The preparation of polystyrene-PEG grafts is a more laborious process compared to the preparation of the present system where no further functionalisation is involved. The new glycerol-based support was found to exhibit an optimum hydrophobic–hydrophilic balance, high mechanical and chemical stability and excellent swelling in various solvents used for SPPS. For a given polymer support the yield, purity and cost effectiveness of a particular synthetic peptide essentially depend on the optimization of various reaction conditions. This paper evaluates the synthesis, optimization of various reaction conditions and utility of a novel PS-TRPGGDA polymer in peptide synthesis. To delineate the role of the macromolecular support in SPPS a systematic study on the nature of the cross-linker, the effect of cross-linking density[14,15] on the swelling and the reaction rate, and the factors affecting the various synthetic steps in peptide synthesis was carried out. A reactivity study of the peptide bond formation reaction was also carried out to find the correlation between the reactivity of the bound functional groups and the structural parameters

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Table 1 Preparation of PS-TRPGGDA resin with various cross-linking densitiess

Cross-linking densities[a] (%)	Amount of styrene/g	Amount of TRPGGDA/g	Yield of polymer/g
1	10.30	0.485	9.28
2	10.21	0.971	10.43
3	10.10	1.46	10.88
4	9.9	1.94	11.22
6	9.79	2.9	12.19
8	9.58	3.882	13.03
10	9.37	4.85	13.83

[a] Cross-linking density here indicates the amount of the cross-linker in the feed in mmol%. The degree of cross-linking is quoted as the proportion of multifunctional cross-linking monomer in the feed polymerisation mixture. Although there is no unambiguous method, and certainly no simple and rapid method, for determining the real cross-link ratio in a polymer network, the nominal figure based on actual feed is a very useful parameter.[14,15]

of the macromolecular matrix. The quantification of these closely related parameters was found necessary for the judicious selection of the optimized reaction conditions.

Results and discussion

Synthesis of PS-TRPGGDA support

Tri(propylene glycol) glycerolate diacrylate cross-linked polystyrene support was prepared by suspension polymerization using benzoyl peroxide as the free radical initiator (Scheme 1). PS-TRPGGDA supports with various cross-linking densities of TRPGGDA (1%, 2%, 3%, 4%, 6%, 8% and 10%) were synthesized by copolymerising the monomers in different ratios (Table 1). The polymer was obtained in beaded shape, as evidenced by scanning electron microscopy

The integrity of the polymer was confirmed by solid-state ^{13}C CP-MAS NMR spectroscopy. The spectrum of the resin in $CDCl_3$ shows an intense peak at 127.79 ppm corresponding to aromatic polystyrene carbons and a peak at 145.034 ppm for the C-3 carbon of the styrene. The peak at 75.012 ppm corresponds to the methine carbon of the cross-linker with secondary hydroxy groups. The peaks at 40.221 ppm and 30.443 ppm correspond to the backbone methylene carbon of the polymer.

The major advantage of the new support is that the hydroxy functional groups are introduced in the polymerization process itself. The secondary hydroxy group present in the cross-linker of the polymer is used for the incorporation of the *C*-terminal amino acid and the target peptide is synthesized by Fmoc or Boc-chemistry. Since the drastic initial functionalisation step is avoided the polymer is highly cost effective. Resins with a hydroxy content of 0.1–0.45 mmol were synthesized by adjusting the co-monomer feed ratio. Excellent control over the degree of functionalisation can be achieved during the polymerization step. Hydroxy group capacities were determined by esterifying the resin with Fmoc-Gly by the MSNT method.[16] The hydroxy functional groups of the resin were quantitatively converted into chloro groups by using $SOCl_2$. The resins were further converted quantitatively into amino resins by the Gabriel phthalimide reaction (Table 2).

Swelling of a resin in a solvent is considered to be a necessary condition, favoring organic chemical reactions in the gel phase. Due to the effect of the hydrophilic cross-linking agent TRPGGDA, the extent of swelling of the resin in solvents of various polarities was found to be twice that of both functionalized and non-functionalized 1% PS-DVB resin (Figs. 1 and 2).

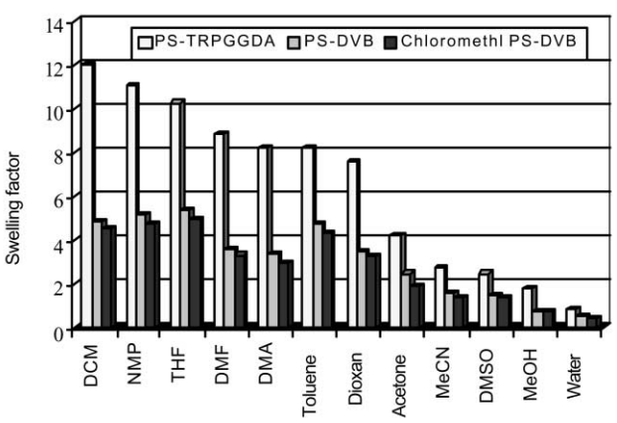
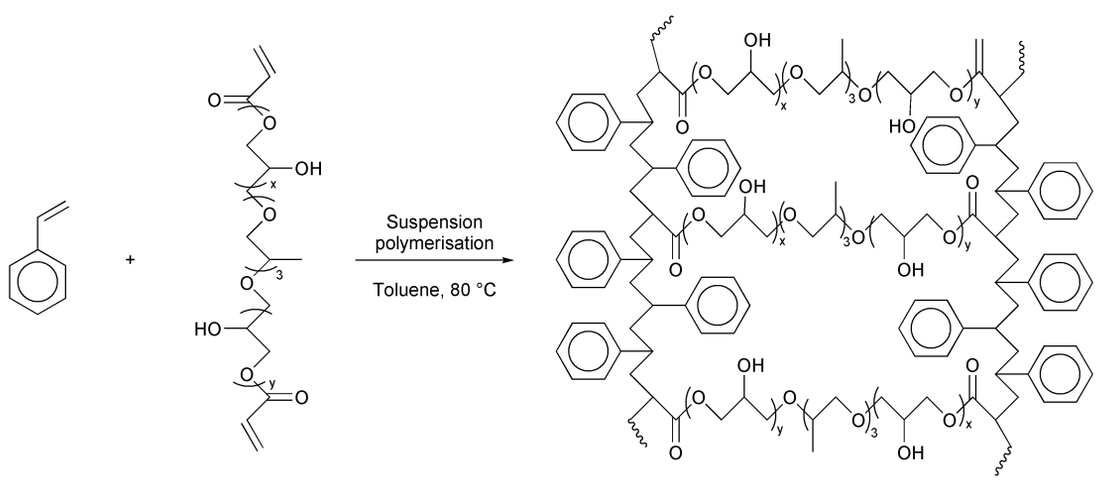


Fig. 1 Swelling comparison between 2% PS-TRPGGDA, 1% PS-DVB and chloromethylated PS-DVB resin in various solvents.

The oxypropylene chains together with ester functionalities and hydroxy groups of the cross-linker confer the optimum hydrophobic–hydrophilic balance to the resin. The principle of optimum hydrophobic–hydrophilic balance is based on the



Scheme 1 Synthesis of PS-TRPGGDA polymer support (*x*, *y* ~ 2–3 glycerolate/TPG).

Table 2 Degree of functional group interconversion of PS-TRPPGDA resin (mmol g^{-1})

Cross-linking density (%)	Hydroxy capacity	Chlorine capacity	Amino capacity
2	0.1	0.1	0.096
3	0.15	0.14	0.13
4	0.20	0.19	0.18
8	0.45	0.43	0.41

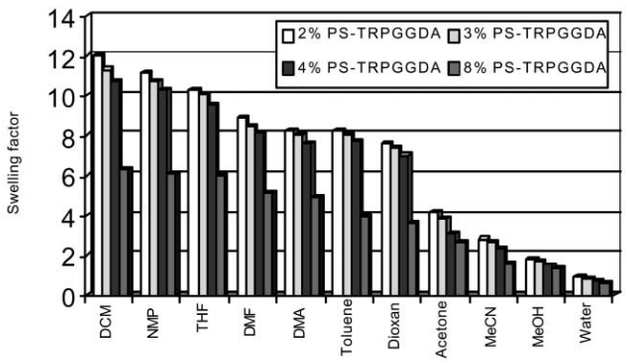


Fig. 2 Swelling comparison between PS-TRPGGDA resins of various cross-linking densities in different solvents.

relative hydrophobicity/hydrophilicity of the resin support and the peptide to be synthesized on the support. This lends compatibility of the resin support to the growing peptide chain. The copolymer was found to be mechanically stable and able to withstand the synthetic manipulations in SPPS. The chemical stability of the polymer was examined by FT-IR spectroscopy and the absence of change in its FT-IR spectrum even after 48 h treatment with various acidic and basic reagents employed in SPPS confirmed its chemical stability.

Optimization of the peptide synthetic cycle

***C*-Terminal amino acid incorporation.** The covalent attachment of a *C*-terminal amino acid to a polymer, the initial step in SPPS, was optimized on PS-TRPGGDA and PS-TRPGGDA–HMPA supports using both Boc and Fmoc-chemistry and compared with PS-DVB and PS-DVB-HMPA supports. The time-dependent attachment of *C*-terminal Boc/Fmoc Val, Ala, Gly, Leu was carried out by monitoring the percentage incorporation into the resin in the presence of MSNT and MeIm in different time intervals. (Fig. 3 and 4). The results showed that for PS-TRPGGDA-HMPA and PS- TRPGGDA-OH resins the quantitative incorporation of *C*-terminal amino acids takes place within 25 min after the addition to the reaction. But under the same reaction conditions the commercially available PS-DVB-HMPA and PS-DVB resins were found to require more than 35 min for the quantitative incorporation of the *C*-terminal amino acid. In the Merrifield system, studies have shown that a spacer molecule attached to the polymer matrix increases the rate of the reaction. In the new system this effect is minimised since the reactants are attached to the flexible cross-linker, thus reducing the steric hinderance caused by the hydrophobic polystyrene core. The higher rate of incorporation of *C*-terminal amino acid in PS-TRPGGDA resin is due to its greater swelling in the reaction medium, which can lead to free interaction between the resin-bound hydroxy functional groups and the activated *C*-terminal amino acids in the medium. Since the secondary hydroxy groups present in the cross-linker are selected for the *C*-terminal amino acid incorporation, the incompatibility between the hydrophobic polystyrene backbone and the activated amino acid in the medium is very much minimised in the swollen state of the resin.

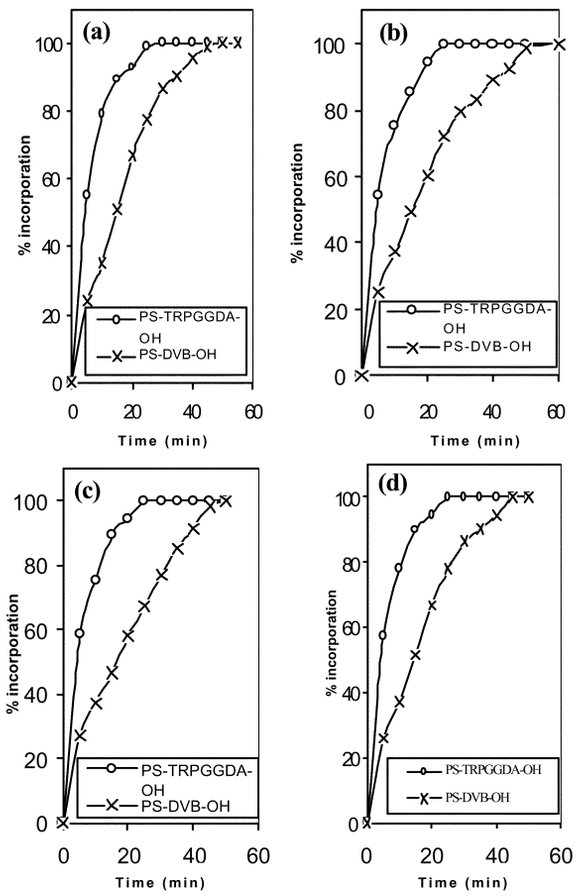


Fig. 3 Time-dependent *C*-terminal amino acid incorporation of (a) Boc-Val; (b) Boc-Gly; (c) Boc-Ala and (d) Boc-Leu.

***N*$^{\alpha}$-Amino group deprotection.** The rate of Boc-deprotection of various Boc-amino acid incorporated PS-TRPGGDA resins were studied and compared with PS-DVB resin under identical conditions by measuring the percentage of the resin-bound free amino groups at different time intervals using the picric acid method.[17] (Fig. 5). The quantitative removal of Boc from the *C*-terminal amino acid was complete within 15 min in PS-TRPGGDA resin whereas PS-DVB resin required 20 min. The efficient permeation of cleavage reagents through the PS-TRPGGDA resin matrix can be attributed to its high degree of swelling in the cleavage reagent mixture and also the balanced polar character of the resin matrix. Swelling studies showed that change of solvent affects the swelling character of both resins (Fig. 6). However, in the case of PS-TRPGGDA, because of the very large extent of swelling compared to commercially available PS-DVB resin in DCM and 30% TFA in DCM mixture, the complete Boc deprotection takes place in a shorter period.

The removal of Fmoc was carried out with 20% piperidine in DMF solution. A comparative study of Fmoc-removal was also carried out using various concentrations (5%, 10%, 15%, 20% and 25%) piperidine in DMF. Time-dependent Fmoc-cleavage studies on PS-TRPGGDA resin showed that 5% piperidine in DMF solution required 22 min where as 25% piperidine in DMF solution required less than 8 min for complete Fmoc-

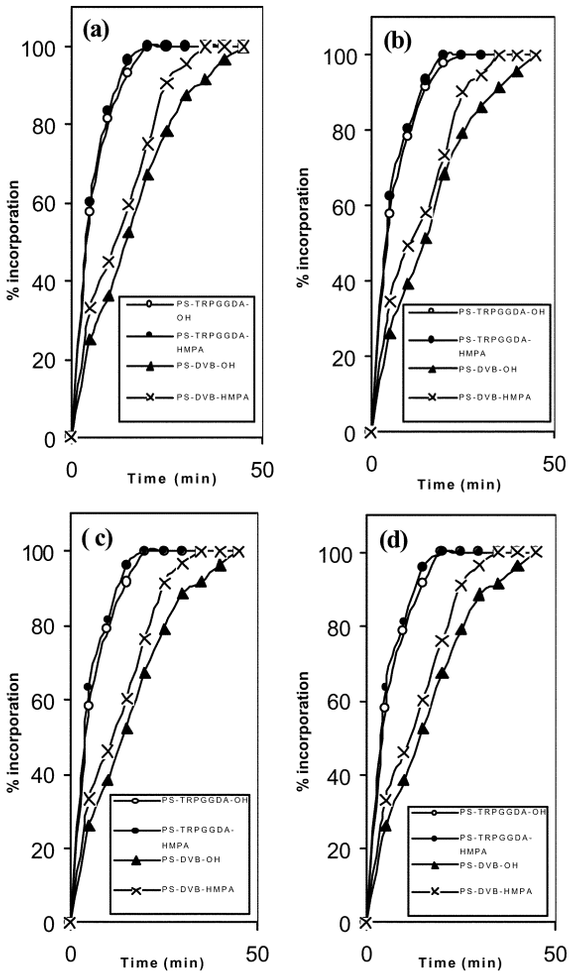


Fig. 4 Time-dependent *C*-terminal amino acid incorporation of (a) Fmoc-Val; (b) Fmoc-Gly; (c) Fmoc-Ala and (d) Fmoc-Leu.

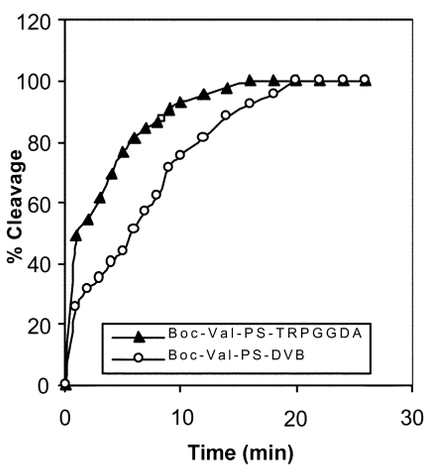


Fig. 5 Time-dependent Boc-removal using 30% TFA.

removal. The rate of cleavage was found to increase with increase in base concentration. To avoid the chance of racemisation and any unwanted side reactions the usual 20% v/v solution of piperidine in DMF was employed throughout the synthesis. Here quantitative Fmoc-removal was found to take place within 10 min of base addition (Fig. 7). The PS-DVB resin took about 18 min for complete Fmoc-removal whereas in the case of PS-DVB-HMPA resin the deprotection time was reduced by 4 min. An Fmoc-cleavage study of both HMPA attached PS-TRPGGDA and PS-DVB peptidyl resins containing six amino acid residues [Val-$(Ala)_4$-Val] was also carried out to study the effect of secondary structure formation of the resin-

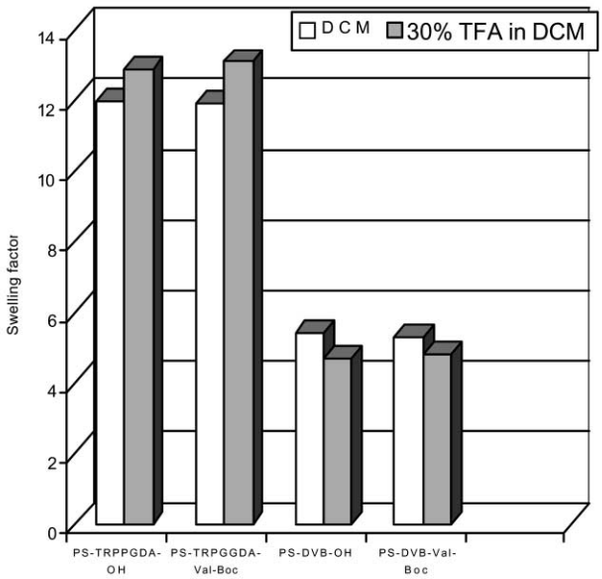


Fig. 6 Swelling studies of resin, N^{α}-Boc-Val resin and peptidyl resin in DCM and 30% TFA in DCM.

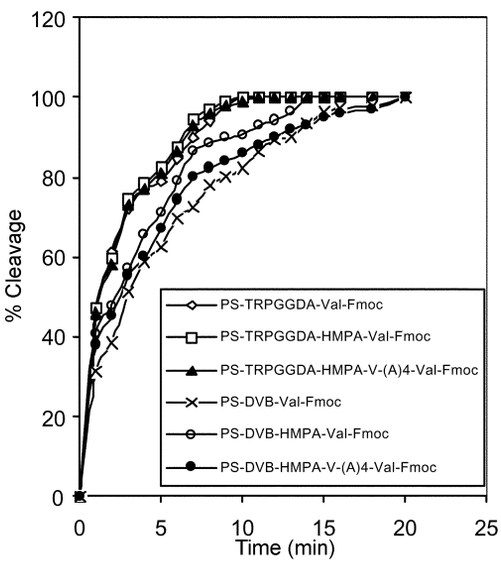


Fig. 7 Time-dependent Fmoc-removal of the *C*-terminal Fmoc-Val from various supports using 20% piperidine in DMF.

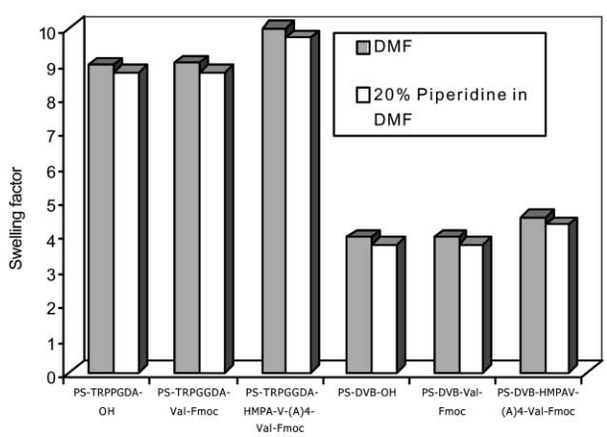


Fig. 8 Swelling studies of the resin, N^{α}-Fmoc-Val resin and peptidyl resin in DMF and 20% piperidine in DMF.

bound peptide in the deprotection step. These studies revealed that the tetra-Ala incorporated PS-TRPGGDA resin had greater swelling characteristics in the deprotection medium and that the rate of cleavage was not affected by the presence of the peptide chain (Fig. 8). These studies revealed the positive role of the

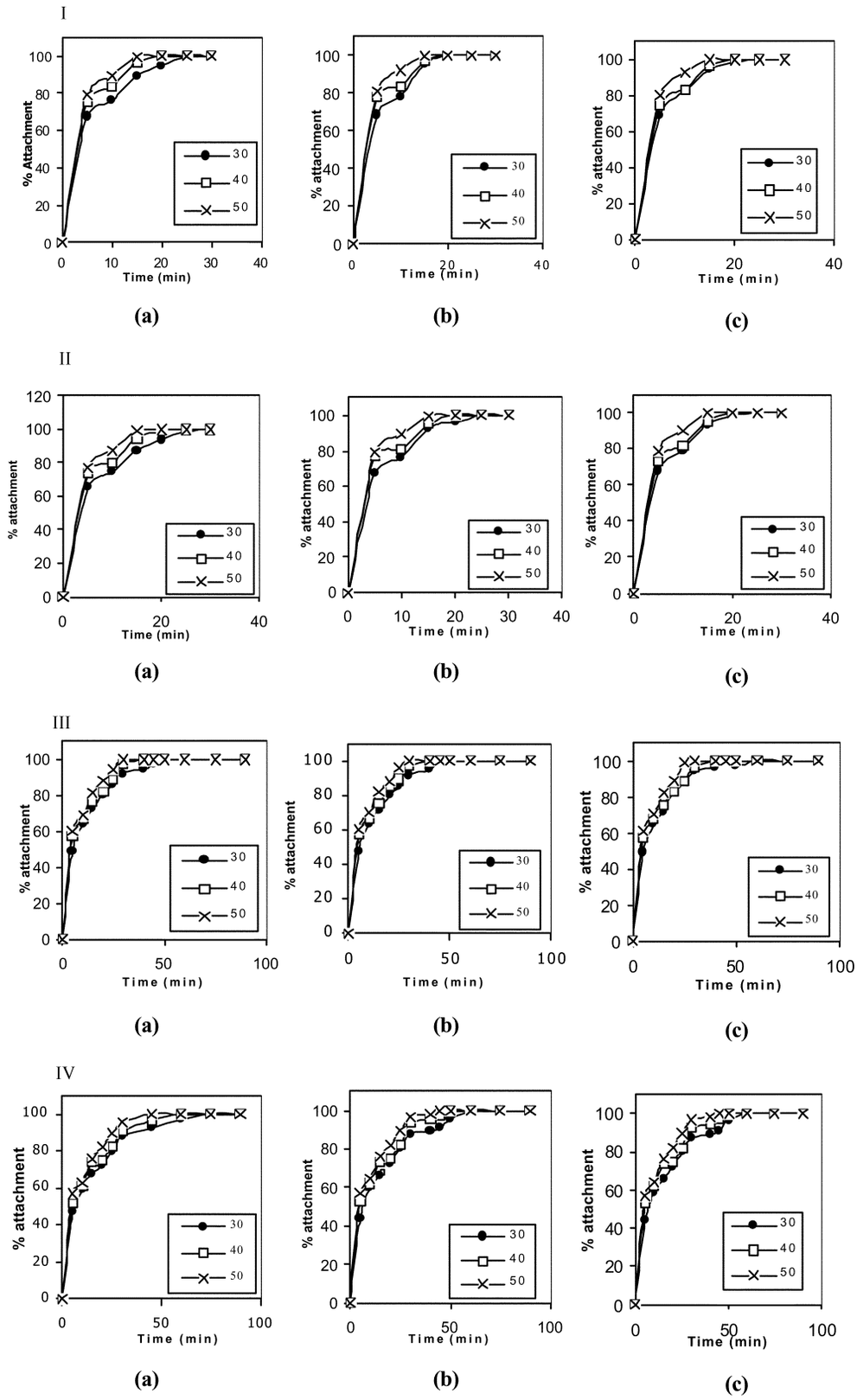


Fig. 9 (i) Time/temperature-dependent coupling: (a) PS-TRPGGDA-HMPA-Val-Gly; (b) PS-TRPGGDA-HMPA-Val-Gly-Ala; (c) PS-TRPGGDA-Val-Gly-Ala-Leu. (ii) Time/temperature-dependent coupling: (a) PS-TRPGGDA-Val-Gly; (b) PS-TRPGGDA-Val-Gly-Ala; (c) PS-TRPGGDA-Val-Gly-Ala-Leu. (iii) Time/temperature-dependent coupling: (a) PS-DVB-HMPA-Val-Gly; (b) PS-DVB-HMPA-Val-Gly-Ala; (c) PS-DVB-HMPA-Val-Gly-Ala-Leu. (iv) Time/temperature-dependent coupling: (a) PS-DVB-Val-Gly; (b) PS-DVB-Val-Gly-Ala; (c) PS-DVB-Val-Gly-Ala-Leu.

TRPGGDA cross-linker in assisting the free interaction between the cleavage mixture and the N^{α}-protected peptidyl resin.

Time and temperature-dependent amide bond coupling. To optimize the PS-TRPGGDA support in amide bond formation, a time and temperature-dependent coupling reaction was carried out using the Merrifield model peptide Leu-Ala-Gly-Val. The results were compared with Merrifield resin under identical conditions [Fig. 9(i)–(iv)]. Each coupling step was compared by adding the respective activated Fmoc-amino acids

to the resin-bound amino acid/peptide and then monitored by the degree of coupling at various time intervals. The time/temperature dependent studies revealed that the amino acid coupling reaction took place at a higher rate when PS-TRPGGDA support was used for the test peptide synthesis. The amide bond formation was complete within 25 min, 20 min, and 15 min in all the different coupling steps in the case of the new support at 30 °C, 40 °C and 50 °C, respectively. Fig. 9(i) and 9(ii) do not show clear asymptotic behaviour at 40 °C and 50 °C. This behaviour is not apparent in polystyrene resins. It is possible that this is the effect of successive amino acid coupling on the temporary swelling of the resin. In PS-DVB-HMPA resin the coupling was complete within 50 min, 40 min and 30 min, and for PS-DVB support within 60 min, 50 min and 45 min, under identical synthetic conditions. The major reason for the low coupling reaction in various stages of peptide synthesis is the steric occlusion of the peptide chain within the polymer network. This in turn depends upon the type of resin and its swelling characteristics in the coupling medium. In solvents like DCM, NMP and DMF, PS-TRPGGDA swells to its elastic limit and creates a space or solvent porosity within the resin which allows ready access of the activated amino acid molecule to the polymer network; this in turn becomes saturated with free-flowing activated amino acid. This can lead to the free interaction between the activated amino acid and the amino terminus of the resin-bound amino acid/peptide. The steric effect, originating from the peptide chain or the sterically hindered amino acid, is extremely low in PS-TRPGGDA resin compared to PS-DVB resin.

Time and temperature-dependent resin-bound peptide cleavage. The time and temperature-dependent cleavage studies of the PS-TRPGGDA supports were conducted using Leu-Ala-Gly-Val peptide and compared with Merrifield resin (Fig. 10).

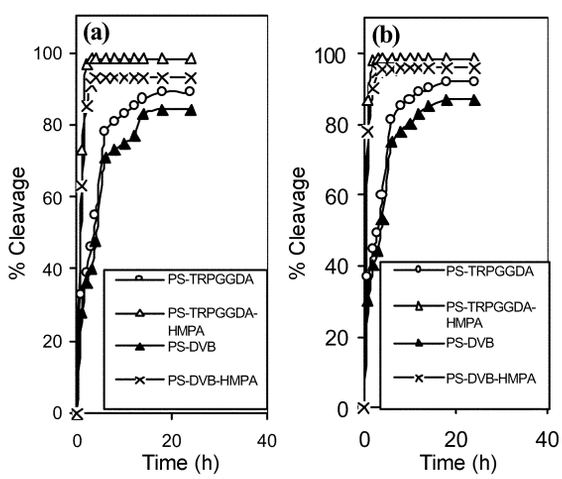


Fig. 10 Percentage cleavage of Leu-Ala-Gly-Val (a) at 30 °C and (b) 40 °C.

The studies revealed that PS-TRPGGDA-HMPA support required 3 h reaction time for 98% cleavage of the peptide at 30 °C whereas the same cleavage yield was found to occur at 40 °C within 2 h. Underivatized PS-TRPGGDA supports required 12 h for 85% cleavage of the bound peptide at 30 °C whereas it required 10 h for 87% cleavage at 40 °C. The studies carried out with Merrifield supports showed that the linker-attached support required 2 h for 90% cleavage at 30 °C and it took 3 h for 91% cleavage at 40 °C. Underivatized Merrifield support required 12 h for 77% cleavage of the bound peptide at 30 °C whereas it required 10 h for 80% cleavage at 40 °C.

Aminolysis of *N*-benzoylglycine 4-nitrophenyl ester by *C*-terminal amino acid attached polymeric supports

To investigate the influence of the nature of the polymer backbone chain on the side chain reactivity in peptide synthesis, a reactivity study of amide bond formation was carried out. In SPPS the time consuming reaction is amide bond formation, as compared to deprotection. Hence monitoring the peptide bond formation assumes more importance than monitoring any other reactions when studying the influence of various properties of the resin on the reaction rate. PS-TRPGGDA-Ala resins of identical functional group capacity were used for the present investigation and a comparative study of the same reaction was carried out with PS-DVB resin. The effects of various cross-linking densities of the resin on the reaction rate of PS-TRPGGDA resin-supported synthesis were also investigated by selecting resins with 2%, 3%, 4% and 8% cross-linking densities. In 2%, 3% and 4% PS-TRPGDDA supports, quantitative conversion of the amino groups was achieved within 12 min (Fig. 11). In PS-DVB only 69% of the reaction was complete even

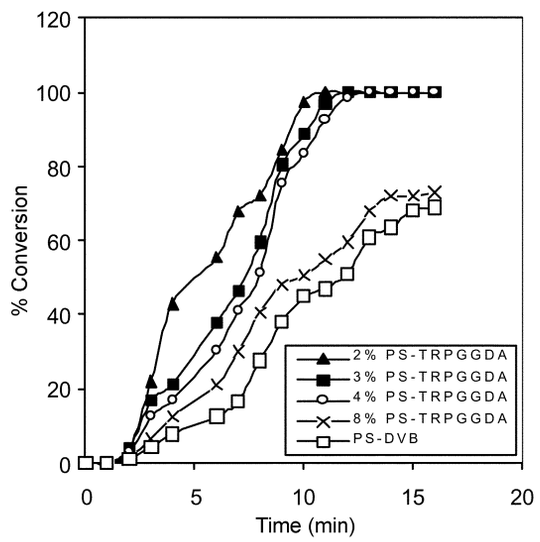


Fig. 11 Reactivity comparison of PS-TRPGGDA with PS-DVB resin.

after 16 min of aminolysis. The slow reaction rate of PS-DVB at the initial stage is because of the slow rate of swelling. In solvents like DCM, DMF and NMP, the PS-TRPGGDA resin network swells to its elastic limits. The amino groups residing inside the matrix are readily available for reaction under these conditions. In the case of the new resin, solvent uptake was very fast and swelling characteristics were greater than in PS-DVB. The differences in reactivity among 2%, 3% and 4% PS-TRPGGDA resin were marginal. Even 8% cross-linked PS-TRPGGDA showed a higher rate of aminolysis compared to PS-DVB resin. These studies revealed a significant influence of the nature and extent of cross-linking on the polymer-supported aminolysis reaction and establish the superiority of the new resin as an effective polymer support for the solid phase synthesis. The enhanced reactivity of the PS-TRPGGDA support is due to the increased availability of amino groups, which are nearer to the hydrophilic core than to the rigid polystyrene core. Whereas in PS-DVB resin the attached reactants are situated close to the rigid polystyrene core, the reactions of this support tend to be affected by the excessive hydrophobicity of the resin. Resins with various cross-linking densities ranging from 2 to 8% PS-TRPGGDA supports were utilised for polypeptide synthesis based on the nature of the sequence to be synthesised and on the requirements of the peptides.

Comparative stepwise synthesis of difficult sequence

The efficiency of the support was established by comparing the synthetic yield and homogeneity of a 'difficult' sequence

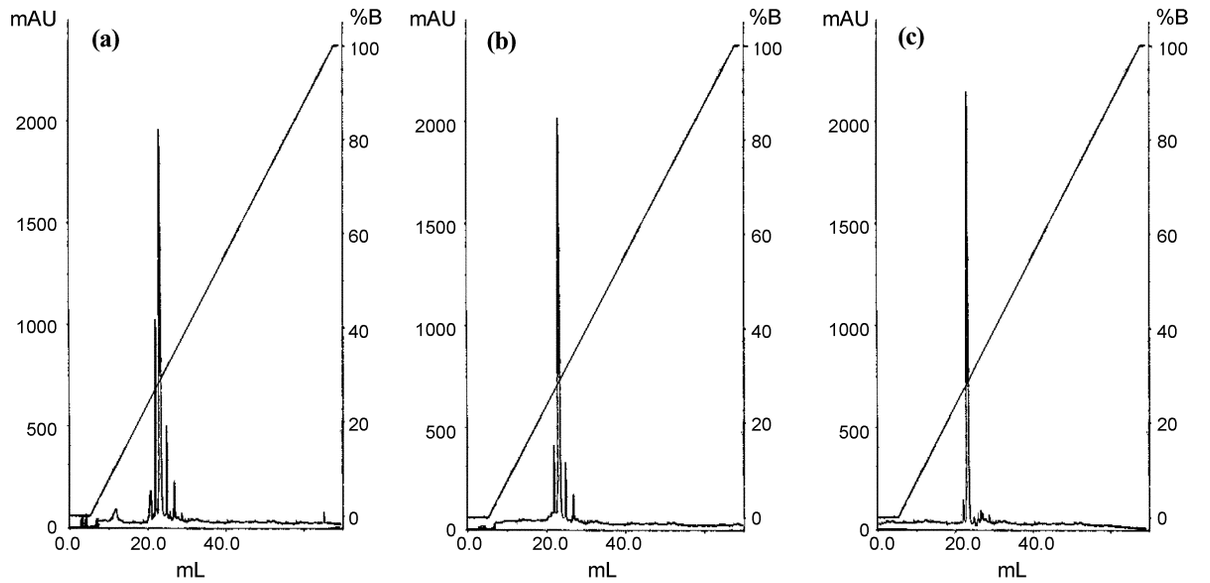


Fig. 12 HPLC time course analysis of β-amyloid peptide (34–42) synthesised on (a) PS-DVB; (b) Sheppard resin; (c) PS-TRPGGDA resin using buffer (A) 0.5 ml TFA in 100 ml water; (B) 0.5 ml TFA in 80% acetonitrile in water.

(34–42) fragment (LMVGGVVIA) of β-amyloid (1–42) peptide with commercially available Merrifield and Sheppard resins. The hydrophobic β-pleated (34–42) fragment of 42-residue β-amyloid protein, implicated in the formation of insoluble plaques in Alzheimer's disease, is difficult to synthesize as it tends to form secondary structures during synthesis, which give rise to sequence-related problems during N^{α}-Fmoc-deprotection and coupling steps.[18] Hence by the synthesis of this difficult peptide the resin behaviour or nature of the polymer/peptide interaction can be revealed. The test peptide was synthesized simultaneously on HMPA handle incorporated PS-TRPGGDA, Merrifield and Sheppard resins. The *C*-terminal Fmoc-Ala was attached to the resin *via* an ester bond using MSNT in presence of MeIm under a nitrogen atmosphere. All coupling reactions were carried out using a 3.5 equiv. excess (with respect to the Ala load) of the respective amino acid, HOBt and HBTU and DIEA. After the synthesis, the peptide was removed from the corresponding resins under same cleavage conditions using Reagent K.[19] The yield of crude peptide obtained from the various resins after a four hour cleavage was 96% from PS-TRPGGDA, 86% from PS-DVB and 92% from Sheppard resin, calculated on the basis of the first amino acid substitution. From the HPLC profile, the peak area corresponding to the target peptide showed that the yield of pure peptide was only 52% in PS-DVB resin and 72% in Sheppard resin, whereas PS-TRPGGDA resin gave a yield of 89% under identical synthetic conditions (Fig. 12). Amino acid analysis and MALDI-TOF-MS confirmed the identity of the target peptide. The enhanced rate of the acylation reaction in the case of PS-TRPGGDA resin reveals the positive role of the flexible cross-linker in the polystyrene matrix, which facilitates the effective solvation of peptidyl resins, thus exposing the peptide chain to soluble reagents. The comparative study indicates the efficiency of PS-TRPGGDA resins in solid phase peptide synthesis.

Solid phase synthesis of 23-residue NR 2B peptide substrate of Ca^{2+}/calmodulin binding peptide Thr-Asn-Ser-Lys-Ala-Gln-Lys-Lys-Asn-Arg-Asn-Lys-Leu-Arg-Arg-Gln-His-Ser-Tyr-Asp-Thr-Phe-Val

The synthetic utility of the polymeric support was further established by synthesizing a 23-residue NR 2B peptide substrate of Ca^{2+}/calmodulin binding peptide. The stepwise synthesis was carried out until the 23-residue target peptide was

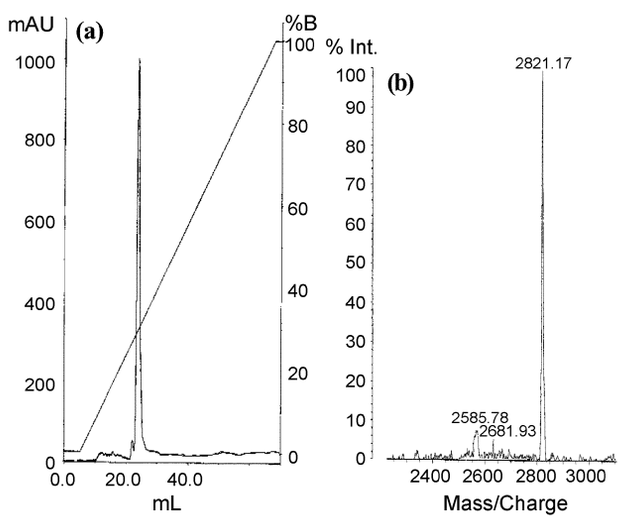


Fig. 13 (a) HPLC time-course analysis of peptide using buffer (A) 0.5 ml TFA in 100 ml water; (B) 0.5 ml TFA in 80 % acetonitrile in water. (b) MALDI-TOF-MS.

achieved, by incorporating the respective amino acid using HBTU, HOBt as the coupling reagents in the presence of DIEA. After the synthesis of the 23-residue fragment of NR 2B, it was cleaved from the support by use of Reagent K. The crude peptide was obtained in 95% yield calculated on the basis of first amino acid substitution. The HPLC profile showed a single major peak indicating the high purity of the NR2B 23-mer peptide (Fig. 13a). The amino acid analysis data agreed with the target peptide sequence. The identity of the target peptide was further confirmed by MALDI-TOF-MS. (Fig. 13b)

Conclusions

This paper reports the synthesis of a novel PS-TRPGGDA support and describes the role of the macromolecular support in SPPS by delineating the optimization of various chemical reaction conditions encountered in this resin during polypeptide synthesis. The support is unique since it avoids the additional chemistry required to convert the polystyrene beads into functional resin, which makes it highly cost-effective. In the new support the increased reactivity is due to the effect of the hydrophilic cross-linker which minimizes the non-compatibility

arising due to the hydrophobicity caused by the polystyrene core. The studies revealed that the swelling and solvation characteristics of the support in a particular solvent are prime factors governing the reactivity of the resin-bound functionality. 2% PS-TRPGGDA support showed maximum solvation in a wide range of solvents employed in the synthesis. The deviation in the swelling characteristices was found to be marginal for 2%, 3%, and 4% cross-linked resins. The effect due to increased cross-linking density is suppressed by the hydrophilic nature of the cross-linker. In the Merrifield system the functional groups are situated close to the hydrophobic core of the cross-linker, and may not be accessible to reagents in continuous phase due to diffusional restrictions. The reactivity studies also revealed that the difference in the reactivity of aminolysis among 2%, 3% and 4% PS-TRPGGDA resin was marginal and even 8% cross-linked PS-TRPGGDA showed a higher reactivity compared to PS-DVB resin. The high yield and purity of the peptides synthesized on the new support reveal the ability of the resin to assist solvation and prevent aggregation of the peptide by direct interaction between the polymer and peptide chain. The results clearly demonstrate that the nature of the polymer backbone is a prime factor, which determines the efficiency of the new support in solid phase peptide synthesis.

Experimental

Materials

Styrene, TRPGGDA, polyvinyl alcohol (M_r ~ 75000), $SOCl_2$, potassium phthalimide, hydrazine hydrate, thioanisole, ethanedithiol, piperidine, and diisopropylethylamine (DIEA) trifluoroacetic acid and *N*-methylimidazole were purchased from Sigma-Aldrich Corp., USA. Solvents (HPLC grade) were purchased from E. Merck (India) and BDH (India). 4-(Hydroxymethyl)phenoxyacetic acid (HMPA), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzatriazole (HOBt) and 1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT) were purchased from Nova Biochem Ltd. UK. FT-IR spectra were recorded on a Bomem MB-series spectrometer using KBr pellets. UV spectra were recorded on a Shimadzu UV 160 A spectrophotometer. The ^{13}C CP-MAS solid-state NMR measurements were conducted on a Varian Unity 400 CP-MAS instrument operating at 100 MHz. HPLC was carried out on a Pharmacia Akta purifier instrument using a C-18 Sephasil peptide reversed phase semi-prep. column. The amino acid analysis was carried out on an LKB 4151 Alpha plus instrument. Mass spectra were recorded on a Kratos MALDI-TOF mass spectrometer.

Synthesis of PS-TRPGGDA polymer

Styrene and TRPGGDA were washed with 1% NaOH solution and distilled water to remove inhibitors. A four-necked reaction vessel equipped with a thermostat, Teflon stirrer, water condenser and nitrogen inlet was used for the polymerization. A net volume of 1% solution of polyvinyl alcohol (M_r ~ 75,000 Da) was prepared by dissolving PVA (1.1 g) in doubly distilled water (110 ml) and added to the reaction vessel. The solution was deoxygenated by bubbling N_2 gas. In a typical polymerization for 2% PS-TRPGGDA support, the monomers, styrene (10.21 g) and TRPGGDA (0.971 g) were mixed with toluene (8 ml) and added to the reaction vessel. The biphasic solution was stirred at a constant rate of 2000 rpm with a thermostatically regulated mechanical stirrer. The radical initiator benzoyl peroxide (0.5 g) was added and the reaction vessel was sealed with a water condenser on one side and a rubber septum on the other. The system was kept under a continuous flow of N_2 gas. The temperature of the reaction mixture was maintained at 80 °C using a thermostatted oil bath and the reaction was allowed to continue for 6 h. The copolymer was obtained as beads and washed thoroughly with hot water (to remove the stabilizer), acetone (3 × 50 ml), toluene (3 × 50 ml) and methanol (3 × 50 ml). The copolymer was further purified by Soxhlet extraction with DCM and MeOH and dried in an oven (P_2O_5, 50 °C) for 10 h to yield 10 g of dry resin.

The hydroxy group capacity of the resins was calculated by esterification with Fmoc-Gly (3 equiv.) activated by MSNT (3 equiv.) in dry DCM in the presence of the base *N*-methylimidazole (2.25 equiv.) and measurement of the UV absorbance of the adduct of dibenzofulvene and piperidine formed by treatment of accurately weighed polymer samples with 20% piperidine in DMF.

Chemical stability studies

The stability studies of the resin were carried out for different reagents such as 100% TFA (10 ml), 20% piperidine in DMF (10 ml), neat pyridine (10 ml), 2 M aqueous NaOH (10 ml), 2 M NH_2OH in aqueous methanol (10 ml) and ammonium hydroxide solution (10 ml). The resin samples (100 mg of each) were separately stirred with the above-mentioned reagents. After 48 h exposure, the respective resin samples were filtered, washed thoroughly with ethanol (3 × 50 ml), water (3 × 50 ml), acetone (3 × 50 ml), DCM (3 × 50 ml), dioxane (3 × 50 ml) methanol (3 × 50 ml) and ether (3 × 50 ml), and dried under vacuum. FT-IR (KBr) spectra of these resins were measured and compared with the spectrum of the untreated resin.

Chlorination

2% PS-TRPGGDA resin (1 g, 0.1 mmol) was suspended in DCM (25 ml). After 1 h, excess solvent was filtered off. To the swollen resin, thionyl chloride (73 µl, 1 mmol) was added. The reaction was heated at 55 °C with occasional swirling for 6 h. The reaction mixture was cooled, washed with THF (5 × 15 ml), THF–H_2O (1:1, 5 × 15 ml), THF (5 × 15 ml), DCM (5 × 15 ml), methanol (5 × 15 ml) and ether (5 × 15 ml), and dried under vacuum. The above procedure was followed for chlorination of resins with higher cross-linking densities; the reactions were carried out using a 10 mmol excess of the reagent with respect to hydroxy capacity of the resin. The chlorine capacity was estimated by Volhardt's method.[17]

Amination

2% PS-TRPGGDA chloro resin (1 g, 0.1 mmol) was allowed to swell in excess NMP for 1 h. The resin was filtered, potassium phthalimide (165 mg, 0.9 mmol) in NMP (20 ml) was added and the reaction mixture was stirred at 120 °C for 12 h. The resin was collected and washed with NMP (5 × 15 ml), dioxane (5 × 15 ml), ethanol (5 × 15 ml), methanol (5 × 15 ml) and ether (5 × 15 ml), and dried under vacuum. The resin was suspended in distilled ethanol (20 ml), hydrazine hydrate (45 µl, 0.9 mmol) was added, and the mixture was refluxed for 8 h. The resin was collected by filtration and washed with hot ethanol (5 × 15 ml), methanol (5 × 15 ml) and ether (5 × 15 ml), and dried under vacuum. Amination was also carried out with 3%, 4% and 8% PS-TRPGGDA support using the procedure described above. The amino capacity of the resin was estimated by the picric acid method.[17]

Preparation of PS-TRPGGDA-HMPA resin

HMPA (64 mg, 0.35mmol), HBTU (132 mg, 0.35 mmol), HOBt (47.29 mg, 0.35 mmol) and DIEA (61 µl, 0.35mmol) were added to pre-swollen amino PS-TRPGGDA resin (1 g, 0.1 mmol) in DMF and the reaction mixture was kept at room temperature for 1 h with occasional swirling. The resin was filtered, washed with DMF (5 × 15 ml), MeOH (5 × 15 ml) and ether (5 × 15 ml), and dried under vacuum. The resin was negative to the sensitive Kaiser test.[20] Derivatization of the amino resin with 4% TRPGGDA was also carried out under the same synthetic conditions.

Time-dependent *C*-terminal Boc-amino acid incorporation

2% PS-TRPGGDA resin (200 mg, 0.02 mmol) was used to study the optimization of the *C*-terminal N^{α}-Boc/Fmoc-amino acid esterification reaction. A comparative study of the same reaction was also carried out using PS-DVB resin (182 mg, 0.02 mmol). Boc and Fmoc-protected Val, Gly, Ala and Leu were the amino acids used for the present study. These amino acids were attached to the support as the active ester using MSNT (2 equiv., 0.04 mmol) in the presence of MeIm (1.5 equiv., 0.03 mmol). The reaction mixture was kept for coupling in a septum-stoppered flask under a nitrogen atmosphere. About 5 mg of the resin were withdrawn from the reaction mixture at 5 min intervals up to 60 min. The aliquot withdrawn was washed thoroughly with DCM (5 × 6 ml), MeOH (5 × 6 ml) and ether (5 × 6ml). The Boc-protection of the *C*-terminal N^{α}-Boc attached resin was removed by treatment with 30% TFA in DCM for 30 min. The resin was then filtered and washed with DCM (5 × 6 ml) and neutralized with 5% DIEA in DCM (3 × 3 ml). The free amino group in the resin was estimated by the picric acid titration method.[17] For monitoring Fmoc-amino acid incorporation: an accurately weighed amount of dried resin (10 mg) was mixed with 3 ml 20% piperidine in DMF for 30 min. The percentage incorporation of amino acid was estimated by measuring the absorbance of the above solution containing dibenzofulvene-piperidine adducts at 290 nm.

Synthesis of PS-TRPGGDA-HMPA-Val-$(Ala)_4$-Val-Fmoc and PS-DVB-HMPA-Val-$(Ala)_4$-Val Fmoc resins

2% PS-TRPGGDA-HMPA-Val-Fmoc (250 mg, 0.025 mmol,) and PS-DVB-HMPA-Val-Fmoc (228 mg, 0.025 mmol) resins were used for the present study. *C*-Terminal amino acid attached resins were allowed to swell in DMF in a manual peptide synthesiser. Fmoc-protection was removed using 20% piperidine solution in DMF (25 ml × 20 min), followed by washing of the resin with DMF (6 × 10 ml). The following sequence of operations was carried out for the introduction of each amino acid residue: (a) washing with DMF (6 × 10 ml), (b) washing with 20% piperidine in DMF (1 × 25 ml), (c) deprotection with 20% piperidine in DMF (1 × 25 ml × 20 min), (d) washing with DMF (6 × 10 ml) (e) acylation, carried out with a 3.5 mmol excess of Fmoc-amino acid, HBTU, DIEA and HOBt relative to the amino capacity of the *C*-terminal amino acid present in the HMPA resin for 40 min. After the incorporation of all amino acids, Fmoc-protection of the *N*-terminal amino acid in the peptide resin was not removed.

Optimization of N^{α}-deprotection studies

Time-dependent Boc-deprotection. Time-dependent Boc deprotection was carried out using Boc-Val-PS-TRPGGDA resin. Boc-Val-PS-DVB was used for a Boc-deprotection comparative study. An amount (200 mg) of these resins was treated with 30% TFA in DCM. About 5 mg of the resin was withdrawn from the reaction mixture in 1 min intervals for 30 min. The Boc-deprotected resin was washed with DCM (5 × 10 ml) and neutralized with 5% DIEA in DCM. The neutralized resin was washed with DCM (5 × 10 ml), MeOH (5 × 10 ml) and ether (5 × 10 ml), and dried. The free amino group in the resin was estimated by the picric acid titration method.[17]

Time-dependent Fmoc-deprotection. Optimization of the time-dependent Fmoc-deprotection was carried out using Fmoc-Val-PS-TRPGGDA resin, Fmoc-Val-HMPA-PS-TRPGGDA and Fmoc-Val-$(Ala)_4$-Val-HMPA-PS-TRPGGDA resin. A comparative study with Fmoc-Val-PS-DVB, Fmoc-Val-HMPA-PS-DVB and Fmoc-Val-$(Ala)_4$-Val-HMPA-PS-DVB resin was also carried out. Fmoc-amino acid attached resin (500 mg) was treated with 20% piperidine in DMF. About 5 mg of resin were withdrawn from the reaction mixture at 1 min intervals for 30 min The Fmoc-cleaved resin was washed with DMF (5 × 6 ml), MeOH (5 × 6 ml) and ether (5 × 6 ml), and dried. Accurately weighed resin was treated with 0.1 M picric acid. The bound picrate was eluted with 5% DIEA in DCM (2 × 3 ml). From the absorbance of the eluate at 358 nm the amino acid capacity and the percentage cleavage were calculated. This was further confirmed by measuring the Fmoc-release at regular intervals of accurately weighed resin by spectrophotometrically monitoring the absorbance of dibenzofulvene adduct at 290 nm.

Optimization of time- and temperature-dependent couplings

2% PS-TRPGGDA-HMPA, 2% PS-TRPGGDA, PS-DVB-HMPA, and PS-DVB (~500 mg, 0.05 mmol) resins were used for the optimization of time- and temperature-dependent coupling studies. The coupling of a model peptide Leu-Ala-Gly-Val was used for the present study. The Fmoc-Val attached resins were deprotected with 20% piperidine in DMF. The resins were washed with DMF (6 × 20 ml). Each coupling step was carried out using HOBt and HBTU as coupling reagents in the presence of DIEA. About 5 mg of the resin were withdrawn from the reaction mixture every 5 min up to 90 min. The resin was washed with DMF (6 × 20 ml), MeOH (6 × 20 ml) and ether (6 × 20 ml), and dried. The Fmoc-content in the resin was measured at regular intervals by monitoring the absorbance at 290 nm of the dibenzofulvene adducts formed. From the absorbance the percentage coupling was calculated. The same protocol was adopted for the reaction at 40 and 50 °C. At elevated temperatures, the peptides were synthesized manually in a water-jacketed vessel connected to a heated water-bath circulator.

Time- and temperature-dependent cleavage of the peptide

Accurately weighed amounts of Leu-Ala-Gly-Val attached 2% PS-TRPGGDA, PS-DVB and their HMPA-attached derivatives were treated separately with TFA (2.85 ml) and water (150 μl) for a time period of 1–24 h at two different temperatures, 30 °C and 40 °C, respectively. The percentage yield of the peptide was calculated by noting the weight of peptidyl resin and the amount of peptide obtained.

Preparation of *N*-benzoylglycine 4-nitrophenyl ester

To a solution of benzoylglycine (1.79 g, 10 mmol) in THF (20 ml), dicyclohexylcarbodiimide (2.06 g, 10 mmol) and 4-nitrophenol (1.39 g, 10 mmol) were added and the reaction mixture was stirred at 0 °C for 60 min. The precipitated dicyclohexylurea was removed by filtration and the solvent evaporated slowly from the filtrate. The residue was washed repeatedly with ethanol to remove free 4-nitrophenol, dissolved in dichloromethane, and precipitated by the addition of petroleum ether.

Aminolysis of *N*-benzoylglycine 4-nitrophenyl ester by *C*-terminal amino acid attached polymeric supports

2% PS-TRPGGDA-Ala-NH_2 (21 mg, 0.002 mmol), 3% PS-TRPGGDA-Ala-NH_2 (16.6 mg, 0.002 mmol), 4% PS-TRPGGDA-Ala-NH_2 (11.8 mg, 0.002 mmol), 8% PS-TRPGGDA-Ala-NH_2 (4.8 mg, 0.002 mmol) and 1% PS-DVB Ala-NH_2 (18 mg, 0.002 mmol) resin beads were used for reactivity studies. A 10 mmol excess of *N*-benzoylglycine 4-nitrophenyl ester (6 mg, 0.02 mmol) in DMF was added to the cuvette containing each of the resin beads. The spectrophotometer was programmed to read the absorbance at regular intervals (1 min) for 20 min. From the absorbance values of *N*-benzoylglycine 4-nitrophenyl ester measured at 275 nm, the percentage peptide bond formation was calculated.

Comparative synthesis of the (34–42) fragment of β-amyloid (1–42) peptide

The synthesis of β-amyloid (34–42) fragment LMVGGVVIA was carried out manually using Fmoc-Ala-HMPA-4% PS-TRPGGDA resin (150 mg, 0.025 mmol), Fmoc-Ala-HMPA-Merrifield resin (135 mg, 0.025 mmol) and Fmoc-Ala-Novasyn^{R} KA 125 resin (180 mg, 0.025 mmol). *C*-Terminal Fmoc-Ala was anchored to these supports using the MSNT method using Fmoc-Ala–MSNT–MeIm in the ratio 2:2:1.5. Fmoc-protection of these amino acid bound resins was removed with 20% piperidine in DMF and the resins were washed thoroughly with DMF (5 × 15 ml). For each acylation cycle the respective Fmoc-amino acids (0.086 mmol), mixed with HBTU (33 mg, 0.086 mmol), HOBt (12 mg, 0.086 mmol) and DIEA (15 μl, 0.086 mmol) dissolved in the minimum amount of DMF, were added to the Fmoc-deprotected resin. Coupling solutions were made at three times the indicated scale and apportioned equally to the three parallel syntheses. The coupling reaction was carried out for 40 min at room temperature. After incorporation of all amino acids, Fmoc-protection of the target peptidyl resin was removed and the resin was washed with DMF (5 × 15 ml), methanol (5 × 15 ml) and ether (5 × 15 ml), and dried under vacuum. The target peptides were cleaved from the polymer supports by suspending the peptidyl resin in Reagent K at room temperature for 4 h. The solution was filtered and the filtrate concentrated under reduced pressure. The peptide was precipitated by adding ice-cold ether and the precipitate was washed with ether until the scavengers were removed, and dried under vacuum. HPLC analysis of the peptide was carried out by injecting a small amount of peptide dissolved in water into a C-18 RPC and eluting using a gradient of solvent A (nanopure water containing 0.5% TFA) and solvent B (80% acetonitrile in nanopure water containing 0.5% TFA). Amino acid analysis of the peptide from PS-TRPGGDA resin: Leu, 1.01 (1); Met, 0.81 (1); Val, 3.02 (3); Gly, 2.02 (2); Ile 0.98 (1); Ala, 1.04 (1). MALDI-TOF-MS: *m*/*z* 859.11 $(M + H)^+$; $C_{39}H_{71}O_{10}N_9S$ requires M^+ 858.09.

Synthesis of NR 2B peptide substrates of Ca^{2+}/calmodulin binding peptide Thr-Asn-Ser-Lys-Ala-Gln-Lys-Lys-Asn-Arg-Asn-Lys-Leu-Arg-Arg-Gln-His-Ser-Tyr-Asp-Thr-Phe-Val

2% PS-TRPGGDA-HMPA resin (350 mg, 0.035 mmol) was used for the synthesis of NR 2B 23-residue peptide substrate of Ca^{2+}/calmodulin binding peptide. The dried resin was allowed to swell in dry DCM (100 ml). After 1 h, excess DCM was removed, and a mixture of the *C*-terminal amino acid Fmoc-Val (24 mg, 0.07 mmol), MSNT (20 mg, 0.07 mmol) and *N*-methylimidazole (8.3 μl, 0.05 mmol) in dry DCM (5 ml) was added. The reaction mixture was kept at room temperature for 30 min and dried under vacuum. The amino capacity of the resin: 0.095 mmol g^{-1}.

Fmoc-Val-HMPA-PS-TRPGGDA resin (325 mg, 0.031 mmol) was placed in a manual peptide synthesiser and allowed to swell in DMF for 1 h. Fmoc group protection was removed with 20% piperidine in DMF (20 ml × 30 min), and the resin was washed with DMF (5 × 15 ml). The remaining amino acids of the target peptide sequence were successively incorporated by using the respective amino acid (0.109 mmol), with HBTU (41.3 mg, 0.109 mmol), HOBt (15 mg, 0.109 mmol) and DIEA (19 μl, 0.109 mmol) for 40 min. All coupling and deprotection steps were monitored by means of the Kaiser test.[20] The resin was washed with DMF (5 × 15 ml), methanol (5 × 15 ml) and ether (5 × 15 ml), and dried under vacuum. Fmoc-protection of the target peptidyl resin was removed and the resin was washed with DMF (5 × 15 ml), methanol (5 × 15 ml) and ether (5 × 15 ml), and dried under vacuum. The NR2B 23 residue peptide was cleaved from the resin by suspension of the peptidyl resin in Reagent K. The solution was filtered and the filtrate concentrated under reduced pressure. The peptide was precipitated by adding ice-cold ether and the precipitate was washed with ether until the scavengers were removed, and dried under vacuum to yield 95% of the crude peptide. HPLC analysis of the peptide was carried out by injection of a small amount of peptide dissolved in water into a C-18 RPC and eluting using a gradient of solvent A (nanopure water containing 0.5% TFA) and solvent B (80% acetonitrile in nanopure water containing 0.5% TFA). Amino acid analysis: Leu, 1.01 (1); Arg, 2.97 (3); Glu, 1.97 (2); Tyr, 0.82 (1); Ser, 1.59 (2); Asp, 3.98 (4); Thr, 1.63 (2); Phe, 1.05 (1), Val, 1.00 (1); His, 0.91 (1); Lys, 4.11 (4); Ala, 1.01 (1). MALDI-TOF-MS: *m*/*z* 2821.17 $(M + H)^+$, $C_{120}H_{199}O_{36}N_{43}$ requires M^+ 2820.06.

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