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Synthetic evaluation of disulphide-bonded sarafotoxin on a poly(oxy ether) grafted dendrimeric poly(alkyl amine) support for polymer assisted organic synthesis†

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The present paper describes the synthesis, characterization and assessment of a novel class of insoluble polymeric polystyrene supports which combines polar poly(ethylene glycol)dimethacrylate as a cross-linker and poly(ethylene glycol) grafted poly(*N*,*N*-bisethylamine) as a dendritic template. Poly(*N*,*N*-bisethylamine) dendrimers were generated by a series of reactions such as Schiff base integration, acidolysis, diazotization and thionyl chloride treatment. The same successive sequences of reactions have been followed for second generation dendrimers also and subjected to PEGylation (PEG 600) to achieve the desirable physico-chemical properties. The applicability of the novel PEGylated dendrimer support was demonstrated by synthesizing linear as well as disulfide bonded peptides in high yields and purities.

Introduction

The concept of solid phase synthesis has its roots in Merrifield's inspiring work on peptide synthesis and has received much consideration in the last few decades.[1] The wide adoption of combinatorial and parallel strategies for compound synthesis in the drug discovery process provided stimulus that has propelled a dramatic increase in the use of solid phase organic synthesis (SPOS).[2,3] These techniques often employ polymeric supports to immobilize either the substrate or the reagent in order to simplify compound manipulation and purification. Insoluble cross-linked polymer supports are most commonly employed for such applications,[4] but soluble polymers have also proven useful.[5] In the case of cross-linked polymer beads, commonly referred to as resins, reactions can be influenced by the accessibility of reagents to reaction sites and the microenvironment associated with the polymer phase within the bead. Characterization of resin-bound compounds and the monitoring of reactions is a critical aspect of successful SPOS.[6] Microporous polystyrene-divinylbenzene (PS-DVB), with its high thermal stability, chemical inertness, mechanical robustness, and various facilities of derivatizations for substrate attachment, substantiates as an attractive solid carrier for organic synthesis, commonly known as Merrifield resin. Despite their widespread use, PS-DVB exhibit limited swelling in highly polar solvents and reduced accessibility of polar reagents into the polymer matrix often gives low-yielding on-bead reactions and, in addition, severely restricts the usage of these polymer supports in aqueous bioassays.[7] In attempts to circumvent these limitations, a variety of large linker units such as poly(ethylene glycol) (PEG) have been attached to PS-DVB supports to optimize their properties for specific applications.[8–11] A diverse approach that has emerged involves the replacement of a rigid cross-linker in polystyrene resins with relatively more flexible molecules.[12–20] The new cross-linkers have been designed both to increase the flexibility of the polymer backbone to allow for better diffusion through the matrix and also to impart a variety of solvent-like properties to the resins. Other types of supports developed and tested over these years for solid phase peptide synthesis (SPPS) include polyamides,[21] cotton and other carbohydrates,[22] poly(ethylene glycol)–poly(acrylamide) (PEGA),[23] and cross-linked ethoxylate acrylate resin (CLEAR).[24]

Upon having a closer look at the polymer assisted organic reactions, the support bound reactions were found to be increasingly complex. The various chemical as well as physical parameters that determine its performance in reactions and other applications are usually the reason for success as well as for failure.[25] So the design of chemically inert and mechanically hard polystyrene supports possessing enhanced swelling and reagent accessibility in reaction media represents a challenge in solid phase technology. The renaissance in supported organic synthesis, driven by small molecule combinatorial approaches to lead generation and optimization, has placed new demands of solid supports.[26] In this direction, we plan to synthesize a new polymeric support by retaining all of the

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beneficial features of polystyrene as back-bone units and combining the flexibility of cross-linkers as well as amphiphilicity of grafted PEG derivatives.

Dendrimers are symmetric, highly ordered, highly branched polymers with a compact spherical structure and unique behavior with potential for a whole range of applications.[27–31] Though most of the dendrimers are synthesized by a conventional solution phase approach,[32,33] it is often challenging requiring long reaction times and non-trivial purification.[34] Solid phase methodology enables reactions to reach completion by using a large excess of reagents. It can also remarkably improve the yield and homogeneity of the formed dendrimer.[35,36] It is also realized that the dendrimeric materials can be functionalized conveniently upon the solid support, giving a route to a whole range of functionalized dendrimers that again benefit from the advantages of solid phase synthesis. So solid phase approaches have drawn much attention during the past few decades[37–39] and a divergent route must be adopted instead of the convergent route adopted in solution phase synthesis. The present work describes the on-resin synthesis of poly(*N*,*N*-bisethylamine) dendrimers to $\mathbf{G_2}$ generation followed by PEG grafting. By dendrimer creation, a higher number of PEG molecules can be accommodated on the polystyrene backbone with minimum steric hindrance.

Results and discussion

Synthesis of PEGylated poly(*N*,*N*-bisethylamine) dendrimers

Initially, a PS-PEGDM-VBC resin was synthesized by suspension polymerization from 2 mol% poly(ethylene glycol)-dimethacrylate (PEGDM ($M_n \sim 330$) as a cross-linker; 2 mol%), styrene (95 mol%) and 4-chloromethyl styrene (VBC, 3 mol%) as backbone units. Dimethacrylate was preferred because of its high chemical stability and a more favorable copolymerization parameter with styrene[40] allows uniform distribution of cross-linking agents throughout the resin matrix. The formation of PS-PEGDM-VBC was characterized by FTIR analysis (Fig. S1†) and shows absorption peaks around 1100 cm^{-1} and 1750 cm^{-1} that correspond to ethereal and carbonyl groups of a cross-linker. Polymer beads having a mesh size between 200 and 400 were collected and used for dendrimer grouping and PEGylation. A chlorine loading of 0.143 mmol g^{-1} was determined by Volhard's estimation method[20] and the spherical geometry was characterized by SEM (Fig. 1a).

The experimental studies revealed that the rate of solvent imbibition, compatibility, solvent/reagent accessibility and synthetic efficiency of cross-linked polymers can be improvised to a greater extent by poly(ethylene glycol) (PEG) grafting to the polystyrene matrix.[41] But at the same time, it was noticed that grafting of PEG molecules significantly reduces the functional loading/gram values. This inadequacy can be overcome by adding a high mole percentage of VBC at the time of polymerization reaction. But introduction of a high concentration of VBC will considerably reduce the grafting efficiency due to steric hindrance generated between the crowded polystyrene backbone and PEG chains. These problems can be impressively overwhelmed by generating dendritic templates on cross-linked polymers so that a higher number of PEG chains can be incorporated with minimum steric hindrance. Also the desirable functional loading values for substrate attachment can be accomplished by grafting low molar mass PEG chains. On this account, the dendritic templates were created up to second $\mathbf{G_2}$ generation by following a series of four stage reactions such as dendron assemblage, acidolytic cleavage, diazotization and thionyl chloride reactions. The dendritic units designed from the Schiff base moiety which was synthesized by an exothermic reaction between diethylenetriamine (en) and benzaldehyde molecules readily form a 1 : 2 bis(benzaldeneiminate)ethylenediamine Schiff base at room temperature. The resulting ligand structure is shown in Scheme 1A and its formation was characterized by ^1H and ^{13}C NMR analysis (data are given in the Experimental section). The various stages of reactions involving dendron grouping to $\mathbf{G_2}$ generation and functional

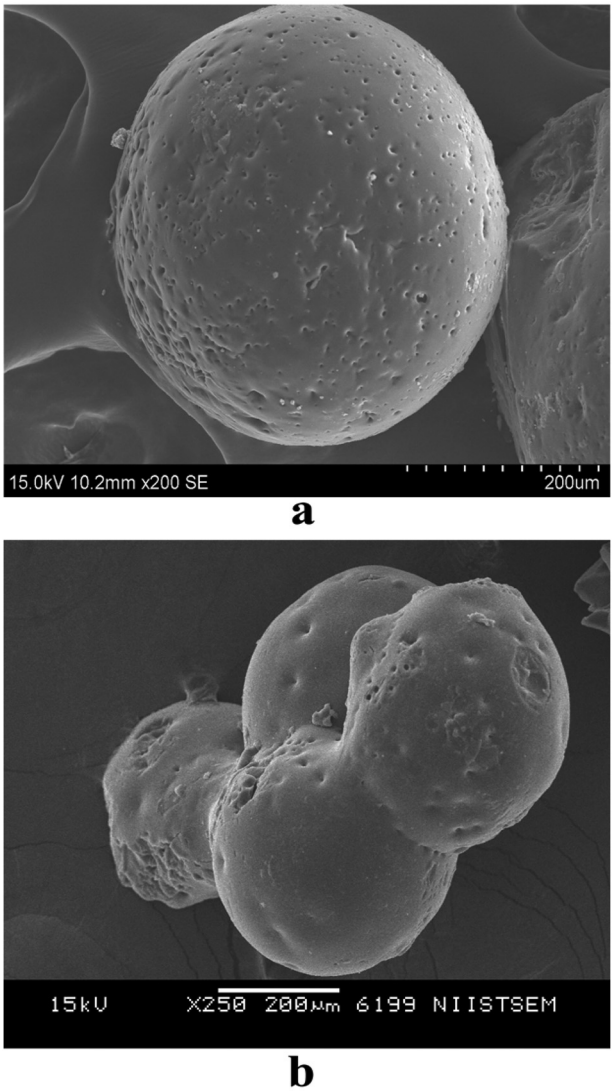
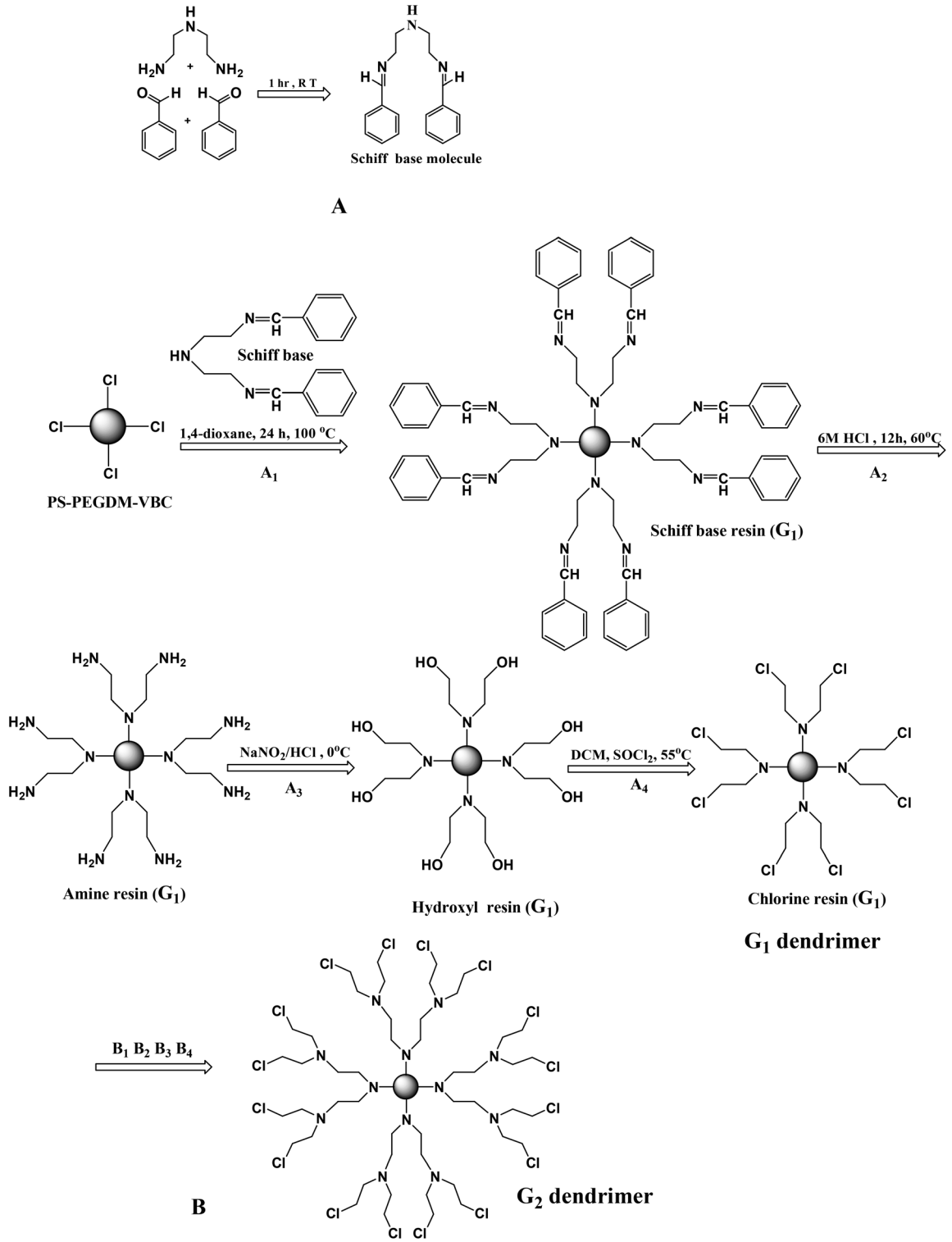


Fig. 1 SEM image of (a) PS-PEGDM-VBC resin, (b) PEGylated $\mathbf{G_2}$ resin.



Scheme 1 (A) Synthesis of Schiff base dendron. (B) Dendrimer synthesis of G_2 generation.

modifications are depicted in Scheme 1B. Since primary amines are more prone to alkylation compared to secondary amines, direct introduction of diethylenetriamine (dien) into chloromethyl groups leads to a wide range of products. So *N*-alkylation specifically through a secondary amine (–NH–) was achieved by synthesizing Schiff base units in which the primary amine groups were intended to protect selectively using benzaldehyde molecules prior to immobilization. The method of Schiff base integration is shown as A_1 in Scheme 1B. The excess of Schiff base and the catalytic amount

Table 1 Functional loading values and % conversion of $\mathbf{G_1}$ and $\mathbf{G_2}$ dendrimers

Gen.	$-NH_2$ (mmol g^{-1}) [UV]	–OH (mmol g^{-1}) [UV]	–OH (mmol g^{-1}) [volumetric]	–Cl (mmol g^{-1}) [volumetric]	% Conversion [using –Cl loadings]
$\mathbf{G_0}$	—	—	—	0.143	—
$\mathbf{G_1}$	0.285	0.283	0.283	0.284	98.60
$\mathbf{G_2}$	0.564	0.563	0.562	0.562	97.88

of trimethylamine which were added during the course of the reaction will function as an acceptor of HCl which was eliminated during the course of *N*-alkylation reaction. Due to protonation, hydrochloride derivatives of free ligands will deposit as yellow crystals which help to maintain the pH of the medium. The light orange colored beads of Schiff base resins formed were collected, thoroughly washed with solvents of various polarities and used for further reactions.

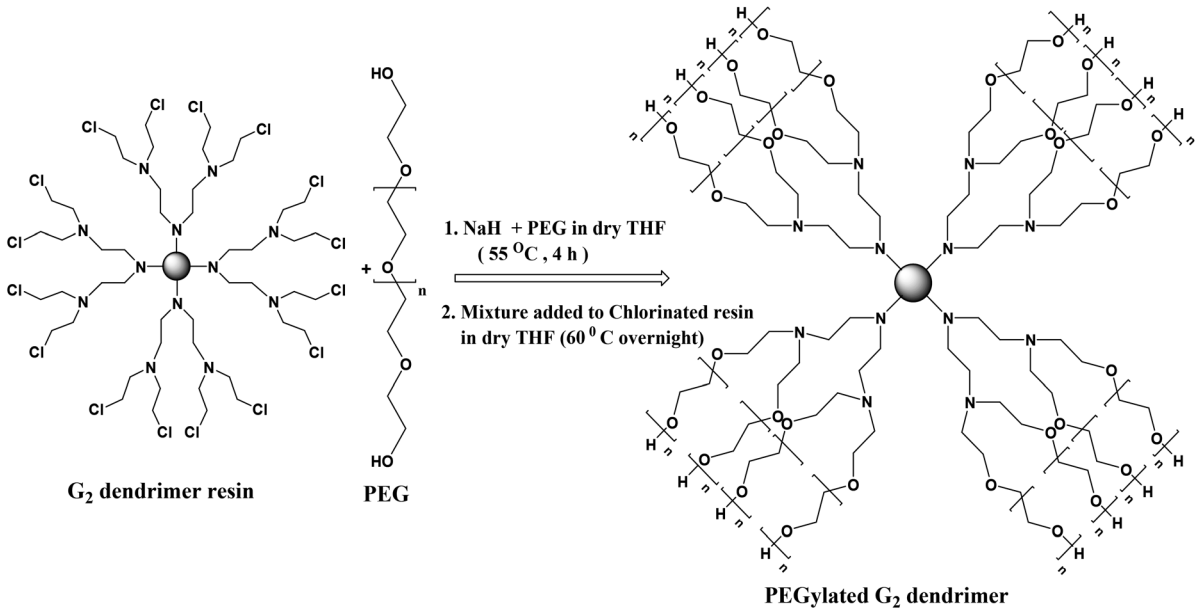
The primary evidence of Schiff base integration was observed by increase in the mass of the resin and was confirmed by FTIR (Fig. S1†) and ^{13}C NMR (Fig. S2†) analysis. The FTIR spectrum of the resin showed an absorption peak around 1640 cm^{-1} that corresponds to –CH═N– linkage of an introduced Schiff base unit and the absence of detectable residual chlorine by Volhard's method. But the inclusion of bulky and hydrophobic aryl moieties eventually leads to intensification in hydrophobicity as well as steric hindrances resulting in undesirable influence on swelling nature and compatibility. Also the imine bond –CH═N– connecting benzaldehyde and dien units was not stable enough to resist the various attacks generated by reagents used for synthetic transformations. So the selective regeneration of primary amino groups of the Schiff base resin ($\mathbf{A_2}$ in Scheme 1B) was achieved by acid hydrolysis[42] and results in $\mathbf{G_1}$ amino resin which showed a positive ninhydrin result. The FTIR spectrum of the amino resin showed an absorption peak at 3450 cm^{-1} due to the regeneration of primary amino groups and amino groups are quantified by the UV method[18] as 0.285 mmol g^{-1}. It was further subjected to diazotization (shown as $\mathbf{A_3}$ in Scheme 1B) and thionyl chloride (shown as $\mathbf{A_4}$ in Scheme 1B) reactions resulted in chlorine terminated *N*,*N*-bisethylamine $\mathbf{G_1}$ dendrimer support. Diazotization was initially checked by a negative ninhydrin test and further evidenced by broadening of the absorption peak around 3450 cm^{-1}. The hydroxyl loadings were quantified by UV[20] and acetic anhydride methods[18] and both methods gave a concordant value of 0.283 mmol g^{-1}. After thionyl chloride reaction, the absorption peak around 3450 cm^{-1} disappeared due to chlorination and –Cl loading was estimated[20] as 0.284 mmol g^{-1}.

The identical reaction conditions and pathways have been followed for the development of a $\mathbf{G_2}$ generation dendrimer. In order to synthesize a $\mathbf{G_2}$ generation dendrimer resin, the chlorinated $\mathbf{G_1}$ dendrimer has been subjected to a series of successive reactions such as Schiff base incorporation, acidolytic cleavage, diazotization reaction and thionyl chloride treatment (shown as $\mathbf{B_1}$, $\mathbf{B_2}$, $\mathbf{B_3}$ and $\mathbf{B_4}$ in Scheme 1B). All stages of $\mathbf{G_1}$ and $\mathbf{G_2}$ reactions and functional modifications were quantitatively analyzed by UV and volumetric methods (Table 1) and confirmed by CHN analysis (Table 2). CHN analysis has been carried out using an amino resin and the loading values have been found using the expression, % $N/14n \times 10$, where % N is the mol percentage of nitrogen obtained from CHN data and '*n*' is the number of nitrogen atoms present in the ligand which is 3. The % transformation of the chlorine atom of each generation by Schiff base ligands was calculated from chlorine and amino loading values and the results obtained are listed in Tables 1 and 2 respectively. In order to find out the % conversion from amino loading values, the Gabriel phthalimide reaction[20] has been carried out on PS-PEGDM-VBC and $-NH_2$ loading was determined[18] as 0.142 mmol g^{-1}.

Table 2 Amino loading values and % conversion of $\mathbf{G_1}$ and $\mathbf{G_2}$ dendrimers from CHN data

Gen.	% Nitrogen (CHN data)	$-NH_2$ (mmol g^{-1}) [CHN]	$-NH_2$ (mmol g^{-1}) [UV]	% Conversion
$\mathbf{G_0}$	—	—	0.142	—
$\mathbf{G_1}$	1.180	0.281	—	97.88
$\mathbf{G_2}$	2.323	0.553	—	96.79

The properties of the polystyrene (PS) support can be modified by grafting PEG onto the resin matrix[39] and proved itself as a spacer for substrate attachment.[43] PEGylated supports are compatible with both polar and nonpolar solvents[44] and are due to the vicinal arrangements of carbon–oxygen bonds throughout the chain which provoke that the PEG assumes helical structures with *gauche* interaction between the polarized bonds.[45] So the optimal desired properties required to perform various organic syntheses were tested by grafting PEG having a molar mass of 600 Da. The lyophilized $\mathbf{G_2}$ dendrimer support was subjected to PEGylation by Williamson's etherification reaction as shown in Scheme 2. The structure of PEGylated dendrimer supports shows change in hydrophobic character due to the most hydrophobic polystyrene backbone, the less hydrophobic dendron mid portion and the least hydrophobic PEG chains on peripheral fringes. These gradients in hydrophobicity make the whole polymer matrix swell extremely well in both polar and non-polar media. The increase in mass after PEG grafting gave a preliminary idea regarding PEGylation and was further confirmed by FTIR, NMR and SEM analysis. The FTIR spectrum (Fig. S1†) showed absorption peaks around 3500 cm^{-1} and 1100 cm^{-1} that correspond to hydroxyl and ethereal groups of grafted PEG. The integrity of PS-PEGDM-VBC, Schiff base and PEGylated resins has been



Scheme 2 Method of PEGylation to the G_2 dendrimer resin.

confirmed by solid state ^{13}C NMR (Fig. S2†). The NMR spectrum of the PS-PEGDM-VBC resin showed an intense peak around 130 ppm that corresponds to all aromatic polystyrene carbon atoms except the one that is linked to the backbone chain. The peaks around 156 ppm (small) and 50 ppm (large) are attributed to *ipso* carbon linked to the aliphatic backbone chain and aliphatic backbone carbon that is linked to phenyl units respectively. Because the second peak is somewhat broad (40–60 ppm), the peak due to methylene groups in the backbone (which is expected to appear at 35–45 ppm) is not separately visible. The Schiff base resin showed a very small peak around 184 ppm that corresponds to imine –CH═N– linkage of the Schiff base which disappeared after acid hydrolysis. After PEG grafting an intense peak at around 83 ppm appears that corresponds to the C–O–C carbon of PEG. PEG grafting was further evidenced by SEM and the result is shown in Fig. 1b. After PEGylation, the rough and porous surface of cross-linked supports became smooth and spongy and the morphological drifting might be attributed to the tethering effects of amphiphilic PEG units. PEG grafting was further supported by reduction in the –OH loading value of 0.403 mmol g^{-1} determined by UV and acetic anhydride methods.

Swelling studies

A useful parameter to determine the applicability of a support for solid phase organic synthesis is its swelling in both polar and nonpolar media. The reactive functional groups in the resin will have the maximum accessibility toward the reactants only when the polymer matrix swells extensively in the solvating medium.[46] So the solvent imbibition ability of 2 mol% PEGDM cross-linked resin, chlorine terminated G_2 and PEGylated G_2 dendrimers was determined by the syringe method

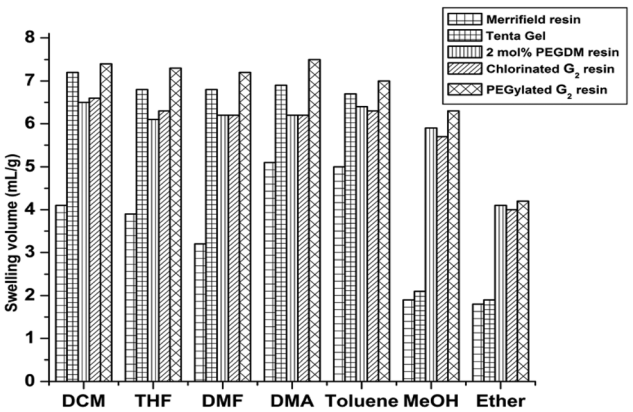


Fig. 2 Swelling comparison of PEGylated dendrimers with PS-PEGDM-VBC, chlorinated G_2, Merrifield and Tenta Gel supports.

and compared to commercially available Tenta Gel and Merrifield resins. The swelling nature has been measured in different polar and non-polar media and the results obtained are summarized in Fig. 2. It has been observed that the PEGDM cross-linked support showed better swelling properties than the Merrifield resin in all solvents. The swelling nature of the chlorine terminated dendrimeric support showed that dendron grouping has no significant influence on solvent uptake ability of cross-linked polymers. A PEGylated dendrimeric system showed substantial variation in swelling nature compared to Merrifield, cross-linked and chlorinated dendrimeric supports. This increase in swelling characteristics might be due to the presence of a large number of grafted amphiphilic PEG chains which imparted better hydrophilicity and solvent compatibility resulting in high hydrophobic/hydrophilic balance towards various reagents and reaction

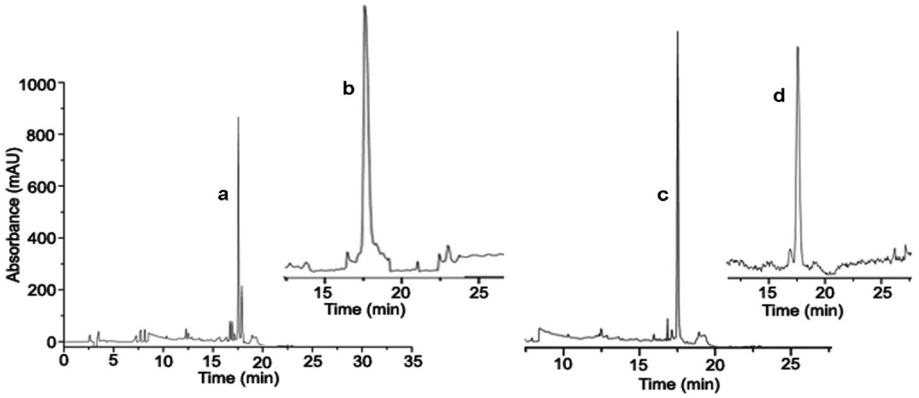


Fig. 3 HPLC profile of retro ACP synthesized on the SAT resin (a) crude form, (b) corresponding purified; dendrimer resin (c) crude form, (d) corresponding purified.

conditions. Compared to Tenta Gel and cross-linked supports, the PEGylated dendrimer support easily attained a 'solvent saturation point' which is a measure of the rate of imbibition in a given solvent within a given time period. These studies revealed that the novel PEGylated support can be very effectively and successfully used as an ideal support for various polymer assisted organic syntheses.

Peptide synthesis

Peptide 1. In order to assess the efficiency of a novel support for polymer assisted synthesis, classically difficult retro ACP fragment [G^{65} N^{66} I^{67} L^{68} D^{69} I^{70} $A^{71}A^{72}$ Q^{73} V^{74}] (65–74) was synthesized on PEGylated and 4 mol% HMPA-SAT supports and the rate of acylation and deprotection reactions compared. SAT was first acylated with HMPA followed by esterification of both supports using first amino acid Fmoc-Trp (Boc)-OH. The first amino acid substitutions were taken as limiting reagent values (0.119 and 0.074 mmol for 300 mg of PEGylated and SAT supports) and used for entire coupling calculations. However, these functional loadings were achieved by giving more reaction time in the case of SAT (90 min, four couplings) compared to the PEGylated resin (45 min, two couplings). The high performance of dendrimeric supports might be due to the presence of a large number of amphiphilic PEG chains which allows fast solvent imbibition and easy diffusion of a reagent–solvent mixture to the resin matrix resulted in ready accomplishment of reaction completion. Further coupling reactions were carried out using 2.5 eq. excess of reagents with respect to the Trp substitution value. The number of couplings as well as the time required for 100% acylation of amino acids and Fmoc removal has been found to be reduced using the PEG grafted dendrimeric support compared to SAT especially for hydrophobic moieties such as His, Phe and Tyr. Using the PEGylated support, single acylation (1 h) was found to be enough for 100% acylation towards Phe, His and Tyr, but minimum of two couplings (1 h each) required for the SAT resin. In most cases of amino acids, Fmoc removal has been finished within 15 min using a novel support compared to SAT which took more than 20 min to obtain the same. The peptides were removed from the supports using a cocktail comprising TFA–H_2O–EDT–TIS (94 : 2.5 : 2.5 : 1 v/v) for 6 h reaction using identical cleavage conditions. The procedures followed for peptide collection and washing were the same as described in the Experimental section. The yields of crude peptides collected for dendrimer and SAT supports were 95.8% and 79.6% respectively. The peptide synthesized was analyzed by HPLC and the results are shown in Fig. 3a–d. The high yield and purity of the peptide synthesized using the PEGylated support might be due to the presence of hydrophilic PEG units which transformed the whole system into highly hydrophobic/hydrophilic balanced in nature. The enhanced compatibility of PEG chains and easy availability of PEG active sites for substrate attachment improvised the rate of diffusion and penetration of reagents to the resin matrix known as the spacer effect. The main peak was collected by the fractional collection method and used for MALDI-TOF analysis to confirm the synthesis. The HPLC chromatograms of purified fractions are summarized in Fig. 3b and d and the corresponding MALDI-TOF are shown in Fig. 4a and b for SAT and dendrimeric supports respectively.

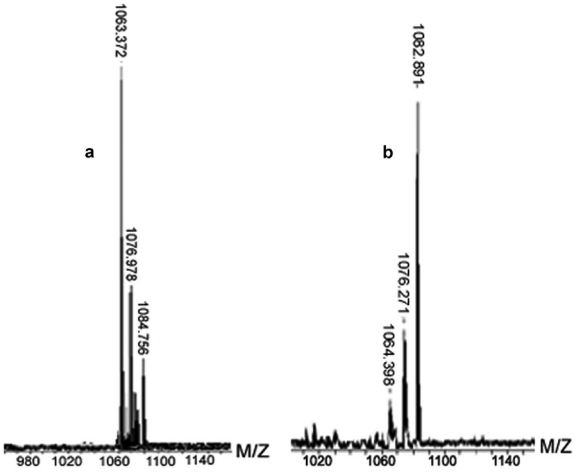


Fig. 4 MALDI TOF of retro ACP synthesized on (a) SAT resin, (b) dendrimer resin.

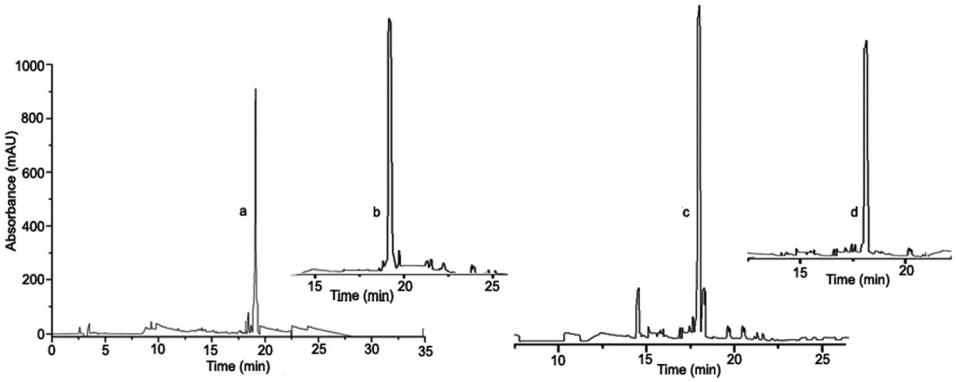


Fig. 5 HPLC profile of peptide 2 (a) after on-resin cyclization (crude), (b) corresponding purified, (c) after air oxidation (crude), (d) corresponding purified.

Peptide 2. A 21-residue peptide Sarafotoxin H_2N-Cys-Thr-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Tyr-Phe-Cys-His-Gln-Asp-Ile-Ile-Trp-OH was synthesized on a PEGylated support. The peptide consists of a C-terminal Trp and two disulfide bonds at positions (1–15) and (3–11) which are responsible for its biological activity. It was synthesized by the combination of on bead[47] and air oxidation methods. The first on-resin intramolecular disulfide formation was carried out between Cys^{1-15} [between Cys(Mmt) and Cys(S-*t*-Bu)] prior to cleavage from the support, followed by second disulfide bond formation between Cys^{3-11} [between Cys(Trt)] by the air oxidation method just after cleaving the peptide from the support. The on-resin cyclization was carried out by the method described in the Experimental section. The disulfide reaction was monitored by absorbance measurement of 5-nitropyridine-2-thione at 386 nm and observed that all the cyclizations were completed within 40 minutes. The peptidyl resin suspended in a cleavage cocktail consisting of TFA–H_2O–EDT–TIS (94 : 2.5 : 2.5 : 1 v/v) for 6 h and was collected by vacuum filtration followed by washing. It was followed by removal of easily volatile components under reduced pressure, chilled ether washing to remove scavengers and lyophilization. The washing of the peptide was performed with the help of Stoppard centrifuge tubes to minimize the exposure of the peptide to the atmosphere to prevent air oxidation. The purity of the single disulfide peptide was checked by HPLC (crude and purified as in Fig. 5a and 5b) and mass by MALDI-TOF analysis (Fig. 6a). The yields of the crude and purified single disulfide bonded peptides were 89.4% and 82.6% respectively which were calculated from the first amino acid substitution value as well as by weighing the dry peptidyl resin. MALDI-TOF of purified peptides did not indicate the presence of sulfur protected Mmt, S-*t*-Bu and Trt groups. This proved the quantitative conversion of disulfide formation by non-oxidative cyclization reaction. Cysteine amino acids present in the 3rd and 11th position will exist as reduced sulfhydryl forms just after cleavage from the polymer matrix which could be readily converted to disulfide bonds in exposing to air by atmospheric oxygen. It was carried out by dissolving the peptide in 0.1 M deaerated ammonium bicarbonate solution and the mixture was left to stand open to the

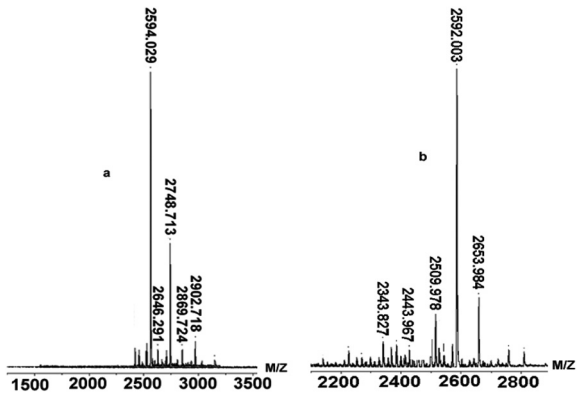


Fig. 6 MALDI TOF of peptide 2 (a) after on-resin cyclization, (b) after air oxidation.

atmosphere until the reaction was completed. In order to avoid the polymeric chain formation, a low concentration of peptide solution (2.5 mg mL^{-1}, 3 mL) has been used and completion of the reaction was monitored by Ellman's test.[48] The double disulfide bonded peptide was lyophilized first and analyzed by HPLC. The HPLC of crude peptide obtained after air oxidation and corresponding purification are shown in Fig. 5c and 5d respectively. The yields of the crude as well as purified disulfide bonded peptides collected were 76% and 69% respectively. The second disulfide bond formation was confirmed by observing the mass of the peptide after oxidation and the result is summarized in Fig. 6b.

Experimental procedure

Materials and methods

Moisture sensitive reactions were performed under an atmosphere of N_2 gas. THF was distilled using sodium in the presence of benzophenone, collected and used. Dichloromethane (DCM) was distilled from anhydrous $CaCl_2$ and poly(ethylene glycol) used was dried well by lyophilization before application. All the swelling experiments were carried out in a plastic syringe equipped with Teflon filters. FTIR spectra were

recorded using a Nicolet 5700 FTIR spectrophotometer. SEM samples were mounted on an SEM sample holder (Hitachi SS 2000) using graphite paint, and a thin coating of gold (10 nm) was applied under high vacuum. Samples were removed and placed in the SEM vacuum chamber and imaged using an accelerating voltage setting of 15 kV. Optical density (OD) values of the dibenzofulvene : piperidine adduct formed were measured with a Shimadzu ultraviolet-visible spectrophotometer at 290 nm. Carbon-13 nuclear magnetic resonance (^{13}C NMR), cross-polarization magic-angle spin (CP-MAS) spectra of the samples were taken using a dsx 300 (75.47 MHz). CHN analysis has been carried out using an Elementar Vario EL III. High performance liquid chromatography (HPLC) was conducted with a Pharmacia Akta purifier system using a C-18 reverse phase semi-preparative HPLC column and a binary gradient system (water and acetonitrile containing 0.1% TFA as the solvents). The flow rate was 1 mL min^{-1}, and detection was at 214 nm. The HPLC conditions used for peptides were the same: C-18 column; buffer (A) 0.1% TFA in water–acetonitrile (19 : 1 v/v) and buffer (B) 0.08% TFA in acetonitrile–water (4 : 1 v/v). Flow rate 1 mL min^{-1}: gradient used 0% B in 5 min, 100% B in 30 min and 100% B in 35 min. Mass spectra of peptides were obtained with a Kratos MALDI TOF MS instrument. All side chain protected F-moc amino acids (L) and 2-(1*H*-benzotriazol-1-yl)1,1,3,3-tetramethyluraniumhexafluorophosphate (HBTU) were purchased from Peptide international company (USA). 1-Hydroxybenzotriazol (HOBt) and 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazol (MSNT) were purchased from Novabiochem Ltd, UK. The following chemicals were purchased from Aldrich (USA) and used without prior purification: styrene, poly(ethylene glycol) dimethacrylate (PEGDM, $M_n \sim 330$), 4-chloromethylstyrene (VBC), polyvinyl alcohol (PVA, $M_n \sim 70\,000$), benzoyl peroxide, thionylchloride, poly(ethylene glycol), sodium hydride, diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), thioanisol, triisopropylsilane (TIS), 1,2-ethanedithiol (EDT), anisol and 1-methylimidazole (MeI).

Suspension polymerization

The PS-PEGDM-VBC support was synthesized by the suspension polymerization method. The experimental method used for polymerization reactions was the same as previously reported.[19] The quantity of reactants used for the preparation of the 2 mol% cross-linked PS-PEGDM-VBC support was as follows: styrene (95 mol%, 10.88 mL), PEGDM (2 mol%, 0.611 mL), VBC (3 mol%, 0.423 mL). The yield of the copolymer collected having a mesh size between 200 and 400 was 5.2 g.

Schiff base preparation

The Schiff base ligand bis[2-(benzaldeneamino)ethyl]amine was synthesized by mixing one equivalent of diethylenetriamine (2.122 mL, 20 mmol) with two equivalents of benzaldehyde (4.062 mL, 40 mmol) dissolved in absolute ethanol at room temperature. An ice cold condition has been provided for half an hour and brought back to room temperature just before reaction completion. After stirring for 60 min, the volume of the solution has been reduced and was rotor evaporated to remove the excess ethanol until only oil remained and was used for the reaction. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 2.0 (s, 1H, –NH), δ = 2.91 (t, 4H, –CH_2, J = 7.1 MHz), δ = 3.6 (t, 4H, –CH_2, J = 7.1 MHz), δ = 7.5 (m, 4H, J = 7.5 MHz), δ = 7.8 (m, 6H, J = 7.5 MHz), δ = 8.65 (s, 2H, ═CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 50.5 (–*CH_2–NH–), δ = 49.6 (–*CH_2–N═), δ = 171 (═*CH–N–), δ = 128, 131, 140 (C_6H_5–).

Schiff base resin

A PS-PEGDM-VBC support (3 g, 0.143 mmol g^{-1}) in 1,4-dioxane (200 mL) was stirred well for 30 min at room temperature to allow full swelling. To this swelled support, suspensions of bis[2-(benzaldeneamino)ethyl]amine Schiff base (2.32 mL, 20 mmol) have been added along with trimethylamine (500 μL) and heated at 100 °C for 24 h with occasional stirring. Excess ligands act as acceptors of HCl and deposited as yellow crystals during the course of the reaction. The solid materials were filtered off, washed well with water (10 × 30 mL) to remove the crystalline compounds and then with dioxane (10 × 10 mL) and transferred to soxhlet apparatus. After continuous extraction of beads with dioxane for 48 h, the resin was collected, washed with ethanol (10 × 20 mL), methanol (10 × 20 mL) and ether (10 × 20 mL) and dried at 50 °C in a vacuum. The yield of Schiff base resin **G$_1$** collected was 3.73 g.

G$_2$ dendrimer synthesis

A **G$_2$** generation dendrimer was developed by following a series of successive reaction paths such as Schiff base incorporation, acidolytic cleavage, diazotization and thionyl chloride addition using a **G$_1$** chloro dendrimer resin (2 g, 0.284 mmol g^{-1}) under identical synthetic conditions followed for the **G$_1$** dendrimer. The quantities of reagents used were as follows: Schiff base reagent (6.08 mL, 52 mmol), 6 M HCl (100 mL), $NaNO_2$–HCl (5 M, 50 mL/2 M, 30 mL) and thionyl chloride reagent (0.816 mL).

PEGylated dendrimer resin

Sodium polyethyleneglycolate (PEG 600) solution was prepared by dissolving a weighed quantity of sodium hydride (270 mg, 11.25 mmol) to PEG (6.24 mL, 11.23 mmol) dissolved in dry THF (100 mL) with occasional stirring. The reaction was allowed to continue till the evolution of H_2 gas formation ceases and kept in an N_2 atm for 4 h. A **G$_2$** chloro dendrimer resin (2 g, 0.562 mmol g^{-1}) was quantitatively transferred to the reaction vessel with the excess addition of PEG (2 mL) and heated at 60 °C for overnight reaction under the continuous stream of inert N_2 atmosphere. The reaction vessel was brought back to room temperature, beads filtered off and unreacted sodium hydride was washed away with distilled ethanol. The PEGylated polymer beads were successively washed with dilute HCl (5 × 15 mL), THF (5 × 15 mL) and ethanol (5 × 15 mL) followed by extraction with THF, methanol

and finally washed with ether (5 × 15 mL). The yield of the PEGylated polymer beads collected was 2.421 g.

Solvent uptake studies

A PEGylated $\mathbf{G_2}$ dendrimer resin (1 g) was accurately weighed and taken in a syringe fitted with a sintered Teflon filter. The solvents were allowed to flow through the resin for 30 min in the syringe with constant suction of a flow rate of 1 mL min^{-1}. The outlet of the syringe was closed and the resin was suspended in the solvent for 1 h. The increase in the volume of the resin at this point was noted and measured in g mL^{-1}. The experiments were repeated to ensure reproducible values at room temperature.

Peptide synthesis

A standard Fmoc strategy has been followed for synthesizing both peptides (peptide 1 and peptide 2). Since PEG will act as a spacer unit which can connect the free carboxyl group of amino acids with the hydroxyl end of the PEGylated resin, first amino acid (2 equiv. excess) can be esterified to the hydroxyl groups of the resin using MSNT (2 equiv. excess) and MeI (1.5 equiv. excess) without preferring any linker. Fmoc protection was removed using 20% piperidine in DMF followed by UV measurements, which gave the amino functional loading value and was used for further calculations of coupling reactions. All the Fmoc-amino acids were coupled to a C-terminal amino acid attached resin by the HOBt active ester method. In a typical coupling step HOBt, HBtU and DIEA (2.5 equiv. each) were added to deprotected Fmoc-amino acid (2.5 equiv.) dissolved in DMF (1 mL). The coupling reaction was continued for 1 h and Fmoc removal was achieved using 20% piperidine in DMF (1 : 4, 3 mL) mixture for 30 min. The extent of coupling and deprotection in all stages of reactions was monitored by the Kaiser test. After each coupling and Fmoc deprotection steps, the resin was thoroughly washed with DMF (5 × 50 mL). After the desired length of peptide has been attached, the peptidyl resin was continuously washed with DMF (5 × 50 mL), MeOH (5 × 50 mL), and ether (5 × 50 mL) and dried in a vacuum. The removal of the peptide from the support was achieved by suspending the peptidyl support in a mixture of cleavage cocktail comprising TFA (4.70 mL), EDT (0.125 mL), TIS (0.05 mL) and double distilled water (0.125 mL) and kept at room temperature for 6 h with occasional swirling. The resin was filtered off, washed with neat TFA (2 × 2 mL) rinsed with dichloromethane (2 × 3 mL), collected and vacuum evaporated to obtain a thick oily residue. The peptide was precipitated as white powder by the addition of ice chilled ether followed by washing thoroughly with cold ether (10 × 10 mL) to remove scavengers. It was dried by lyophilization and used for HPLC and MALDI-TOF analysis. If the samples were impure, pure fractions were collected by the fractional collection method and used for further analysis. The yield of the peptide obtained was predicted by comparing the weight of the peptidyl resin and the amount of the peptide obtained just after cleavage and purification. The quantity of the peptide formed was also calculated by comparing the mass of the dried peptide collected after cleavage with theoretical mass which was calculated on the basis of the first amino acid substitution value.

Peptide 1. Synthesis of retro ACP peptide, G^{65} N^{66} I^{67} L^{68} D^{69} I^{70} $A^{71}A^{72}$ Q^{73} V^{74} (65–74), was carried out using weighed quantities of the PEGylated dendrimer resin (300 mg, 0.121 mmol –OH loading) and previously reported linker attached 4 mol% HMPA-SAT resin (300 mg, 0.075 mmol –OH loading) under identical synthetic conditions. The first C-terminal attachment was carried out using a mixture of Fmoc-Trp(Boc)-OH–MSNT–MeI (2 : 2 : 1.5 equiv. excess) in the molar ratio of 1 : 1 : 0.75 in dry DCM (3 mL each). Complete incorporation of first amino acid was quantitatively verified by spectrophotometric measurement of the adduct dibenzofulvene-piperidine formed and the amino loading values obtained were in agreement with the initial hydroxyl loading values and used for further calculations. Further coupling reactions were carried out using a mixture of Fmoc-amino acid–HoBt–HBtU–DIEA (2.5 equiv. excess each) in DMF. The removal of peptides from solid supports and further washing procedures were the same as described in the general protocol.

Peptide 2. The disulfide bonded Sarafotoxin peptide H_2N-Cys-Thr-Cys-Lys-Asp-Met-Thr-Asp-Lys-Glu-Cys-Leu-Tyr-Phe-Cys-His-Gln-Asp-Ile-Ile-Trp-OH was synthesized on PEGylated dendrimer supports (300 mg, 0.121 mmol) taken in a glass peptide synthesizer. The peptide consists of two disulfide bridges connecting between Cys^3 and Cys^{11} [Cys^{3-11}] and Cys^1 and Cys^{15} [Cys^{1-15}]. The first C-terminal Fmoc-Trp-(Boc)-OH was attached to a pre-swelled dendrimer resin using a mixture of MSNT–MeI. The 1st and 15th cysteine residues were Fmoc-Cys(Mmt)-OH and Fmoc-Cys(S-*t*Bu)-OH which will undergo non-oxidative on-resin intramolecular cyclization before cleaving from a support and the thiol protecting groups used were 4-methoxytrityl (Mmt) and *tert*-butylthio (S-*t*Bu). The 3rd and 11th cysteine residues were protected with Fmoc-Cys-(Trt)-OH which is kinetically stable in piperidine and labile in TFA and undergoes cyclization by the air oxidation method after detaching the peptide from the support. The coupling reactions using all cysteine residues were performed twice to achieve quantitative conversion. In the first disulfide bond formation step, S-*t*-Bu was removed by reduction to liberate free thiol by treating the peptidyl resin with 20% mercaptoethanol in DMF (2 mL) for 3 h. The resin was then reacted with a 5-fold excess of 2,2′-dithiobis(5-nitropyridine) in dichloromethane (12 mL) for 1 h and the free thiol was thus reprotected and activated with the 5-nitropyridinesulfenyl (5-Npys) group. This was followed by the cyclization step in which the resin was treated with 1% TFA in dichloromethane (4 mL) in the presence of TIS (40 μL) as a scavenger. The reaction was monitored by measuring the absorbance of 5-nitropyridine-2-thione at 386 nm. The resin was washed with DMF (3 × 15 mL), dichloromethane (3 × 15 mL), ether (5 × 5 mL) and then dried well. After detaching the single disulfide bonded peptide from the support using a cleavage cocktail, the filtrate was vacuum evaporated at 40 °C to obtain a thick oily residue. The peptide was precipitated as a white powder by

adding ice-cold ether. The precipitate was washed thoroughly with cold ether (5 × 10 mL) to remove the scavengers which were collected and lyophilized. The second disulfide bond formation was carried out by the air oxidation method in which the peptide was dissolved in a 0.1 M solution of de-aerated ammoniumbicarbonate solution (2.5 mg mL^{-1}). The mixture was kept open to the atmosphere with occasional stirring and the completion of the reaction was monitored by Ellman's test. The peptide was dried by lyophilization and used for HPLC and MALDI analysis.

Conclusions

The present work detailed the synthesis, characterization and evaluation of the PEGylated alkylamine $\mathbf{G_2}$ dendrimer for polymer assisted peptide synthesis. A novel class of PEGylated dendrimeric support was designed in such a way as to facilitate substantial compatibility and fast diffusion of a solvent–reagent mixture to the resin bound active sites for substrate attachment. Low initial loading polymers were preferred to avoid all the difficulties customarily encountered due to steric hindrance offered between the bulky nature of Schiff base molecules and crowded polystyrene backbone chains. The Schiff base dendron bis[2-(benzaldeneamino)ethyl]amine was designed in such a way that primary amino groups of diethylenetriamine were intended to be protected by benzaldehyde units prior to immobilization to the support. A series of chemical transformations such as acidolytic cleavage, diazotization and thionyl chloride reactions lead to $\mathbf{G_1}$ generation dendrimers. The same successive reaction paths have been followed for site enlargement to $\mathbf{G_2}$ dendrimers. PEGylation to the $\mathbf{G_2}$ generation resulted in balanced physico-chemical parameters such as swelling, enhanced compatibility and improved reaction rates. Overall, PEGylation transformed the polystyrene matrix to a highly hydrophobic/hydrophilic balanced network and displayed commendable properties throughout the synthesis. The favorable solvation and swelling characteristics of the support facilitated effective synthesis and fast accomplishment of reaction completion. The properties of the polymer matrix can be tunable by regulating initial chlorine values, the number of dendrimeric generations and by choosing PEG having different chain lengths. The chemical structure of the novel support delivered admirable synthetic properties and reagent/solvent accessibilities in different polymer assisted reactions. The classically difficult retro ACP and biologically potent Sarafotoxin peptides were synthesized in high yields and purities and it was demonstrated that a novel PEGylated dendrimer resin can be effectively and successfully used for synthesizing a hydrophobic, highly structured and synthetically challenging peptide series. So the PEGylated dendrimeric support offers tremendous advantages over merely flexible cross-linked polystyrene supports for a broad range of synthetic applications which comprises all the advantages of cross-linked as well as PEG grafted resins. We expect that the present resin will be useful for a wide array of SPOS applications and we are currently exploring the scope of their utility for various organic reactions as well as for combinatorial library assays.

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