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ORIGINAL ARTICLE

PEGylated dendrimer polystyrene support: synthesis, characterisation and evaluation of biologically active peptides

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Abstract Poly(*N*,*N*-bisethylamine) dendrimers with high content of poly(ethylene glycol) were synthesized on 3-(Acryloyloxy)-2-hydroxypropylmethacrylate-crosslinked polystyrene (PS-AHMA) resin and tested in various conditions of solid phase peptide synthesis. The dendritic templates were generated to the second generation on cross-linker active site of 3-(Acryloyloxy)-2-hydroxypropylmethacrylate (AHMA). First generation dendrimer was designed by series of four-stage reactions, such as Schiff base incorporation, acidolytic cleavage, diazotization and thionyl chloride treatment and same synthetic routes were followed for second generation also. Poly(ethylene glycol) (PEG1000) has been grafted to second-generation dendrimer and used to check various physico-chemical parameters in Fmoc/Boc peptide synthetic conditions. The utility of PEGylated dendrimer support was demonstrated by synthesizing biologically potent linear as well as disulfide-bonded peptide by Fmoc method.

Keywords Dendrimer · Solid phase synthesis · Poly(ethylene glycol)

Introduction

Polymer supported organic synthesis improvised extensively to efficiency, speed, and flexibility in synthetic chemistry (Coin et al. 2007; Geysen and Schoenen 2003) and has become an important tool for the progress of preparation of bioactive molecules, especially high-value pharmaceuticals (Pierre et al. 2003). The nature of polymer matrix, solvent polarity, and reactivity of reagents possesses various chemical and physical parameters that determine its performance in reactions and other applications. Therefore, selection of polymeric matrix plays decisive role for the success of reactions (Labadie 1998) and preferred which are chemically inert to a broad range of reaction conditions and applicable in many solvents of different polarity. Until recently, the insoluble polymer resin of choice has been the one originally introduced by Merrifield (1963), divinylbenzene cross-linked polystyrene (DVB-PS), and are commercially available in different functionally derivatized forms. However, its swelling performance in polar media was severely restricted due to the presence of high hydrophobic macromolecular network of short and rigid cross-linker DVB connecting the polystyrene (PS) backbone. This resulted in increased support heterogeneity with consequent deleterious effects upon reaction kinetics leads to reduced accessibility of polar reagents into the polymer matrix, often gives low-yielding on-bead reactions, and eventually restricts the usage of support in aqueous bioassays (Guyot and Bartholin 1982). The combinatorial library synthesis and drug discovery processes have sparked the quest for efficient novel supports (Kumar et al. 2004). Therefore, to rectify the inherent difficulties associated with PS-DVB, different strategies that include replacement of DVB by more flexible cross-linkers (Dolle et al. 2005; Wang et al. 2006; Roice and Pillai 2005; Krishnakumar and Mathew 2002; Siyad et al. 2010; Jacob et al. 2008; Siyad and Kumar 2012a, b) were followed to impart a variety of solvent-like properties to the resins and to allow better diffusion of solvents and reagents through the matrix. Properties of the polymers can

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be tailored by varying the amount and the length of the cross-linking unit. In contrast, a support based on using poly(ethylene glycol) as the major component of resin can be more compatible with aqueous solution as first demonstrated with PEGA (Auzanneau et al. 1995). Because many organic reactions such as glycosylation (Schleyer et al. 1997) were not compatible with the amide functionalities present in the PEGA resin, several new resins having poly(ethylene glycol) as the main content (Rademann et al. 1999) were developed. An alternative approach to impart polar solvent/aqueous compatibility was to graft larger linker units such as poly(ethylene glycol) (PEG), directly onto the polystyrene support (Zalipsky et al. 1994). Among these PEG-grafted polystyrene supports, Tenta Gel has been used extensively in solid-phase synthesis because of the mechanical stability of the beads and the swelling properties in organic and aqueous media (Rapp et al. 2002). Argo Gel displays similar characteristics to Tenta Gel and swells more extensively because of a higher PEG content (Gooding et al. 1999). The flexible PEG grafts have been used to provide a solution-like environment to the resin matrix as well as for bound molecules being synthesized (Wang and Yang 2010).

One of the principle limitations of synthesis of polymer molecules using available resins is the non-quantitative binding of high molecular weight species (Lu and Felix 1994) because of the inaccessibility of functional groups used for conjugation. This unfavorable conditions are often found in areas where the active functional groups are well buried in matrix core as well as in micro-domains where crowding of functional sites happens. Hence, quest for an ideal support for multistep polymer synthesis should possess isolated functional sites located in an amphipathic environment within each polymer beads. This minimizes the unfavorable interaction between the growing moieties, which in turn reduces the chance for deletion and truncation of products. In this focus, we have synthesized and reported new polymer support, styrene-acryloyloxyhydroxypropyl methacrylate-tripropyleneglycol diacrylate [SAT] (Siyad et al. 2010) where synthesis can be initiated and propagated from well-isolated cross-linker functional sites rather than from sterically hindered backbone units. In addition to functional groups carrying cross-linker acryloyloxyhydroxypropyl methacrylate, SAT comprises of polar tripropyleneglycol diacrylate to enhance the amphiphilicity and mechanical strength of polymer beads. The present work describes the modification of amphiphilicity of SAT resin by the introduction of PEGylated dendritic units to the cross-linker active sites. The enhanced rate of conjugation and quantitative conversions can be achieved in two ways: (a) extensive swelling where buried functional sites are well exposed to reaction media and reagents, (b) lengthening of 'active group' carrying chains to the reactive sites in which free mobility and reorganization of chains will result in high rate and fast completion of reaction known as spacer effect. Therefore, the present work describes the increasing polarity of 3-(Acryloyloxy)-2-hydroxypropylmethacrylate crosslinked polystyrene (PS-AHMA) PS-AHMA resin by generating PEGylated chemically robust dendritic templates to second generation so that highly amphipathic, well-isolated and chemically well-defined dendritic units can be used for substrate attachment with improved hydrophilicity with minimum steric hindrance.

Materials and methods

General

All commercial grade solvents were purified and solid chemicals were dried well before application. DCM was distilled using anhydrous $CaCl_2$ and THF using metallic sodium. All side chain protected F-moc amino acids (L) and 2-(1H-benzotriazol-1-yl)1,1,3,3 tetramethyluroniumhexafluorophosphate (HBTU) were purchased from Peptide international company (USA). 1-Hydroxybenzotriazol (HOBt) and 1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazol (MSNT) were obtained from Novabiochem Ltd., UK. Styrene, 3-(acryloyloxy)-2-hydroxypropylmethacrylate (AHMA), polyvinyl alcohol (PVA) (M_n ~70,000), benzoyl peroxide, thionyl chloride, diethylenetriamine (dien), benzaldehyde, trimethylamine, poly(ethylene glycol) (PEG), sodium hydride, diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), triisopropylsilane (TIS) and 1-methylimidazole (MeI) were purchased from Aldrich Chemical Company, USA. Infrared (IR) spectra of polymer samples were recorded using Shimadzu IR 470 spectrometer. Optical density (OD) values were measured with a Shimadzu UV–Visible spectrophotometer at 290 nm. CHN analysis was carried out using Elementar Vario EL III apparatus. ^{13}C NMR; cross-polarization magic-angle spin (CP-MAS) spectra of the samples were taken using a dsx 300 (75.47 MHz). High performance liquid chromatography (HPLC) analysis was carried out using a Pharmacia Akta purifier system using C-18 reverse phase semi preparative HPLC column and binary gradient system (water and acetonitrile) containing 0.1 % TFA as the solvents. The flow rate 1 mL/min and detection was at 214 nm. The HPLC conditions used for all synthetic peptides were 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: gradient used 0 % B in 5 min 100 % B in 30 min and 100 % B in 35 min. SEM pictures photographed using Hitachi SS 2000 scanning electron microscopy. Mass spectra of peptides were analyzed with a Kratos MALDI-TOF MS instrument.

Micro scale suspension polymerization of PS-AHMA resin

Destabilized monomer (styrene, 96 mol %, 10.99 mL) and cross-linker (AHMA, 4 mol %, 0.752 mL) were mixed with porogen toluene (8 mL) and radical initiator benzoyl peroxide (0.5 g) was dissolved in it. The mixture was suspended in a PVA stabilizer solution ($M_n \sim 70{,}000$, 110 mL) at 90 °C and stirred at a rate of 1,400 rpm for 8 h under a stream of N_2 gas. The mixture was cooled to room temperature and the polymeric products formed were collected by filtration. The collected bead polymer was continuously washed with hot water (50 × 50 mL) followed by solvent extraction with toluene, DCM and MeOH, and dried to a constant mass. The mesh size 100–200 was collected and used for further studies.

PS-AHMA-Chloro resin

4 mol % PS-AHMA resin (0.252 mmol/g, 4 g) was allowed to swell in distilled DCM (100 mL) for 1 h. The excess DCM was filtered off and $SOCl_2$ (0.733 mL, 10 mmol excess) reagent was added at 55 °C in dropwise manner with occasional swirling. After 3 h, the resin was filtered off, washed with DCM, DMF, dioxane, ethanol, and methanol (10 × 30 mL each), and dried in vacuum. The amount of chlorine replaced was quantified by standard Volhard's method and obtained as 0.249 mmol/g.

Schiff base dendrimer ($\mathbf{G_1}$) synthesis

Bis[2-(Benzaldeneamino)ethyl]amine synthesis

The Schiff base ligand was formed by dissolving one molecular equivalent of dien (1.061 mL, 10 mmol) with two molecular equivalent of benzaldehyde (2.0312 mL, 20 mmol) in absolute ethanol at room temperature. After 1 h stirring, the volume of the solution has been reduced, rotor evaporated and used for reaction without further purification. The formation of ligand was analyzed by ^{1}H NMR. ^{1}H-NMR (400 MHz, $CDCl_3$) $\delta = 2.0$ (s, 1H,–NH), $\delta = 2.91$ (t, 4H, $-CH_2$, J = 7.1 MHz) $\delta = 3.6$ (t, 4H, $-CH_2$, J = 7.1 MHz), $\delta = 7.5$ (m, 4H, J = 7.5 MHz), $\delta = 7.8$ (m, 6H, J = 7.5 MHz), $\delta = 8.65$ (s, 2H, = CH).

Schiff base incorporation

To the pre-swelled PS-AHMA-Chloro ($\mathbf{G_0}$) resin (2 g, 0.249 mmol/g) in 1,4-dioxane, suspension of bis[2-(Benzaldeneamino)ethyl]amine Schiff base (2.32 mL, 40 mmol) was added with trimethylamine (0.5 mL) and heated at 100 °C for 24 h with occasional swirling. The excess Schiff base ligands act as an acceptor of hydrogen chloride and deposit as yellow crystals. The unreacted materials were filtered off, washed well with water to remove the crystalline compound and then with dioxane (5 × 10 mL) and transferred to solvent extraction apparatus. After extraction with dioxane for 48 h, the Schiff base resin was collected, washed with ether (5 × 10 mL) and dried in 40 °C under vacuum. The yield of resin collected was 2.48 g.

Diethylene triamine (Dien) resin

The Schiff base G_1 resin (2 g) was allowed to stir with HCl (6 M, 100 mL) at 70 °C for 12 h. The liberated benzaldehyde forms an oily emulsion during the hydrolysis and yellow polymer beads obtained were the hydrochloride form of the resin, which were filtered off, washed with ethanol, ether, and dried under vacuum. The amine resin formed was suspended in 0.5 M sodium hydroxide solution (50 mL) followed by thorough washing with water until the solution was turned neutral. Dried well and the free amino groups formed were qualitatively analyzed by positive ninhydrin test. The free amino groups formed were quantitatively analyzed by acylation with Fmoc-Gly-OH followed by measurement of UV absorbance of the adduct dibenzofulvene:piperidine formed at 290 nm. The amino loading obtained was 0.495 mmol/g.

Diazotisation reaction

The amino $\mathbf{G_1}$ resin (2 g, 0.495 mmol/g) was taken in a R.B flask and added 2 M HCl (20 mL) and kept at 0 °C for 15 min stirring. To this resin, 5 M sodium nitrite solution (30 mL) was added in dropwise manner with constant stirring. After the addition of sodium nitrite solution, the reaction was allowed to stand for 1 h in cold condition and brought back to room temperature. The hydroxyl resin beads were washed with excess hot water (5 × 10 mL), 2 M NaOH solution (3 × 10 mL), ethanol (5 × 10 mL), methanol (5 × 10 mL), and ether (5 × 10 mL) and qualitatively checked by negative ninhydrin test. The hydroxyl groups formed were quantitatively analyzed by esterification with Fmoc-Gly-OH and UV absorbance measurements at 290 nm. It was further confirmed by volumetric estimation of free acetic acid molecules formed by reaction with acetic anhydride. Both methods gave concordant value of 0.494 mmol/g.

(e) Hydroxyl to chloro groups: Hydroxyl resin (2 g, 0.494 mmol/g) was suspended in double-distilled DCM (25 mL) and allowed to swell for 1 h. The excess solvent was filtered off and thionyl chloride (73 μL each, 10 mmol excess) has been added in dropwise with occasional swirling and kept at 55 °C for an overnight reaction. The reaction mixture was successively washed with DCM

(5 × 15 mL), THF (5 × 15 mL), THF/H_2O (1:1, 5 × 10 mL), methanol (5 × 15 mL), ethanol (5 × 15 mL) and ether (5 × 15 mL) and dried under vacuum. The chlorine loading value was calculated by Volhard's estimation as 0.493 mmol/g.

Synthesis of $\mathbf{G_2}$ dendrimer

The chlorine-terminated first generation $\mathbf{G_1}$ dendrimer was used for the synthesis of $\mathbf{G_2}$ dendrimer by following identical synthetic reaction paths, such as Schiff base incorporation, imine bond breakage, diazotization reaction, and chloro-resin formations. All the stages of the reactions were qualitatively and quantitatively analyzed. The functional loading values calculated for different transformations are as follows: $\mathbf{G_1}$ chloro-resin (2 g, 0.493 mmol/g) was subjected to Schiff base incorporation (4.64 mL, 80 mmol), acidolytic cleavage ($\mathbf{G_2}$) (–NH_2 loading, 0.983 mmol/g), diazotization reaction ($\mathbf{G_2}$) (–OH loading, 0.982 mmol/g) and thionyl chloride treatment ($\mathbf{G_2}$) (–Cl loading, 0.981 mmol/g). The pre-synthetic condition, time duration and post-synthetic treatments used for $\mathbf{G_2}$ generation development were same as that used for $\mathbf{G_1}$ dendrimer synthesis.

PEG1000 grafting to $\mathbf{G_2}$ dendrimer

A solution of sodium polyethyleneglycolate was prepared by adding weighed quantity of sodium hydride (12 mg, 0.5 mmol) to calculated amount of PEG (5.960 g, 2.98 mmol) dissolved in dry-THF until a green color persisted and stirred for 15 min; weighed amount of well-dried $\mathbf{G_2}$ chlorinated resin (1.5 g each, 1.49 mmol) was added to this solution and heated to reflux for 12 h under an atmosphere of N_2 gas. The polymer beads were washed thoroughly with dilute HCl (5 × 5 mL), ethanol (5 × 15 mL), methanol (5 × 15 mL), DCM (5 × 15 mL), acetone (5 × 15 mL) and ether (5 × 5 mL) and further solvent extracted with THF. The PEGylated resin was further washed with ether (5 × 5 mL) and dried in vacuum to constant weight. The yield of dendrimer resin collected was 2.131 g. The hydroxyl loading value was quantitatively analyzed by UV method and obtained as 0.491 mmol/g.

Swelling studies

Resins (1 g) were placed in a 15-mL syringe equipped with a 0.45 μm filter, treated with enough solvent to swell the resin, and allowed to stand for 30 min. The swollen resin was compressed with the piston until no more solvent could be extracted. The piston was pulled slowly until the resin recuperated its maximum volume in the syringe, and the volume of the resin was read (the void volume of the tip and the syringe was averaged to 0.15 mL). The swelling was calculated according to the following formula: (volume of the swelled resin + 0.15 mL) = x (mL/g). A deviation of <10 % was noted for each solvent and for each resin.

Chemical stability studies

Chemical inertness toward various peptide synthetic conditions was examined by exposing PEG-grafted resin to different reaction conditions and reaction times. Therefore, the chemical inertness of the support was tested by treating with neat TFA at 30 °C (12 h). Apparently, the cross-linked resin and dendritic templates were stable enough to withstand the TFA activity. The stability of resin was further confirmed from the identical IR spectra of TFA treated and untreated resin. The stability of resin was tested by various basic peptide synthetic conditions also; 20 % piperidine in DMF (reagent used for Fmoc deprotection) for 6 h and with aqueous 2 M NH_2OH and 2 M NaOH (12 h each). The stability of support was further checked by treating with 30 % TFA in DCM (the reagent used for Boc deprotection) for 12 h duration.

Polypeptide synthesis

Peptide 1

The human endothelin peptide having amino acid sequence NH_2-Ile–Ile-Trp-Phe-Asn-Thr-Pro-Glu-His-Val-Val-Pro-Tyr-Gly-Leu-Gly-Ser-Pro-Arg-OH (peptide 1) was synthesized on HMPB-SAT (4 mol %, 500 mg, –OH loading: 0.249 mmol/g) and PEGylated dendrimer resin (4 mol %, 500 mg, –OH loading: 0.491 mmol/g) by Fmoc method under identical synthetic conditions. The first amino acids were anchored to free hydroxyl groups of both supports by esterification using Fmoc-Arg(pbf)-OH:MSNT:MeI mixture (1:1:0.75 molar ratio) in dry DCM (3 mL each). Fmoc groups were deprotected using 20 % piperidine:DMF mixture (3 mL) and the extent of reactions were monitored by Kaiser Test. The first amino acid substitution values were determined by UV measurements and obtained as 0.244 and 0.488 mmol/g for SAT and dendrimer supports, respectively, and used for further calculations. All the remaining Fmoc amino acids were coupled to C-terminal end of first amino acid anchored resins using equimolar mixture of HOBt:HBtU:DIEA (1.5 excess). After each coupling and deprotection steps, the resins were thoroughly washed with DMF (5 × 50 mL each). When desired sequence of amino acids were attached to both resins, they were washed thoroughly with DMF (5 × 50 mL each), MeOH (5 × 50 mL each), and ether (5 × 10 mL each)

and dried under vacuum. The lyophilized peptidyl resins were suspended in a mixture of cleavage cocktail comprises of TFA (4.75 mL), TIS (125 μL), and double-distilled water (125 μL). The mixtures were kept at room temperature for 6 h. Both supports were filtered off, washed with fresh TFA, rinsed with DCM, and vacuum evaporated to obtain thick oily residues. The peptides were precipitated as white powder by addition of ice-cold ether, and washed thoroughly with cold ether (10 × 10 mL) to remove the scavengers. The peptides were dried well by lyophilization and used for analysis. The yields of peptides formed were calculated by comparing the weights of peptidyl resin before and after synthesis as well as from first amino acid substitution values.

Peptide 2

ET_A receptor antagonist with amino acid sequence H_2N-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile–Ile-Trp-OH having disulfide linkage between Cys^1 and Cys^5 was synthesized on weighed quantity (250 mg, 0.121 mmol) of first amino acid Fmoc-Trp(Boc)-OH anchored PEGylated dendrimer support. After attaching the desired length of amino acids, an intra-molecular on bead disulfide bond formation was carried out by reported protocol (Galande et al. 2005). The Fmoc-Cys-OH amino acids in which thiol protecting groups used were 4-methoxytrityl (4-Mmt) for first and tert-butylthoio (S-tBu) for fifth positions, respectively. Well-dried peptidyl resin (250 mg) was treated with 2-mercaptoethanol in DMF (1:4, 3 mL) for 3 h. Filtered off and washed the resin with DMF (5 × 15 mL) and DCM (5 × 10 mL). Resin was further treated with excess volume (30 mL) of saturated solution of 2,2′-dithiobis(5-nitropyridine) (DTNB) in DCM for 1 h. This was followed by the cyclization step in which resin was treated with 1 % TFA in DCM in presence of TIS (250 μL TFA:50 μL TIS, 5 mL). The reaction was monitored by measuring the absorbance of 5-nitropyridine-2-thione formed at 386 nm and all the cyclizations were completed within 30 min. The removal of peptide from support and washing were same as described for Peptide 1.

Results and discussion

Initial phase of work consist of synthesis of various cross-linking densities (2, 4 and 6 mol %) of beaded 3-(acryloyloxy)-2-hydroxypropyl methacrylate (AHMA) cross-linked polystyrene, abbreviated as PS-AHMA, by standard aqueous suspension copolymerization method. Beads having diameter 150–250 mesh size have been sieved, collected, and used for entire studies such as dendrimer developments, PEG-grafting and peptide synthesis. Swelling of a gel-like resin is considered a prerequisite for facilitating reactions to occur within the solid support (Groth et al. 2001). However, resins with low cross-linking density have insufficient mechanical stability and swell excessively result in increased amount of solvent and reagents to promote efficient synthesis. From swelling assay (described in swelling studies), it was found that 4 mol % PS-AHMA swelled to a greater extent than other two cross-linking densities and have considerable functional loading value of 0.251 –OH mmol/g and hence chosen for further studies. The –OH capacity of 4 mol % PS-AHMA was quantified by acetic anhydride method and confirmed by UV measurements (Siyad et al. 2010). The cross-linker hydroxyl to chlorine groups were carried out by the dropwise addition of thionyl chloride reagent so that Schiff base dendrons units can be specifically and uniquely bonded to cross-linker by *N*-alkylation reaction. The formation of initial PS-AHMA and chlorinated resins ($\mathbf{G_0}$) were qualitatively analyzed by FTIR (Fig. 1). The initial resin PS-AHMA shows peak around 1,720 and 3,490 cm^{-1} correspond to carbonyl and hydroxyl groups of cross-linker in addition to the usual peaks of phenyl rings and aliphatic hydrocarbon chains. After thionyl chloride addition, the peak near 3,490 cm^{-1} disappeared indicating chlorination reaction. The chlorine functional loading has been quantitatively analyzed by Volhard's estimation method and obtained as 0.249 mmol/g and was in accordance with initial hydroxyl loading value estimated. The *N*,*N*-bisethylamine dendron units were generated on cross-linker active sites to second

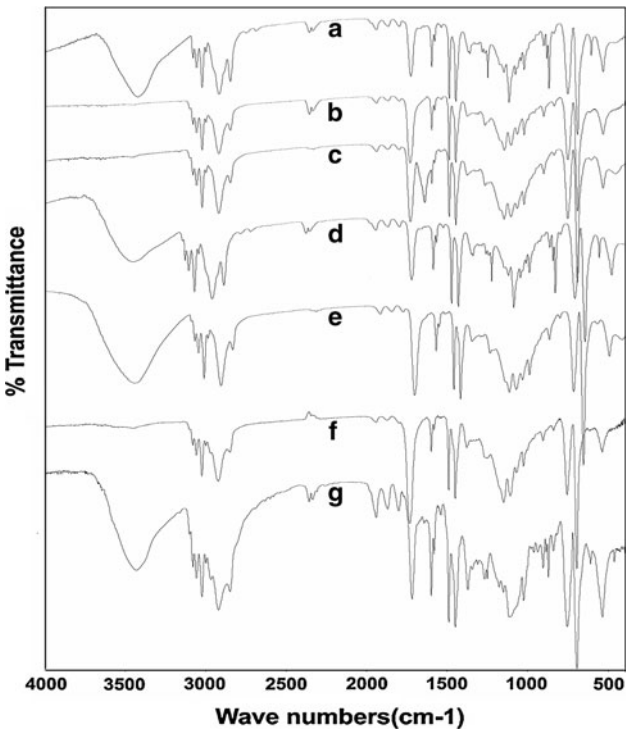


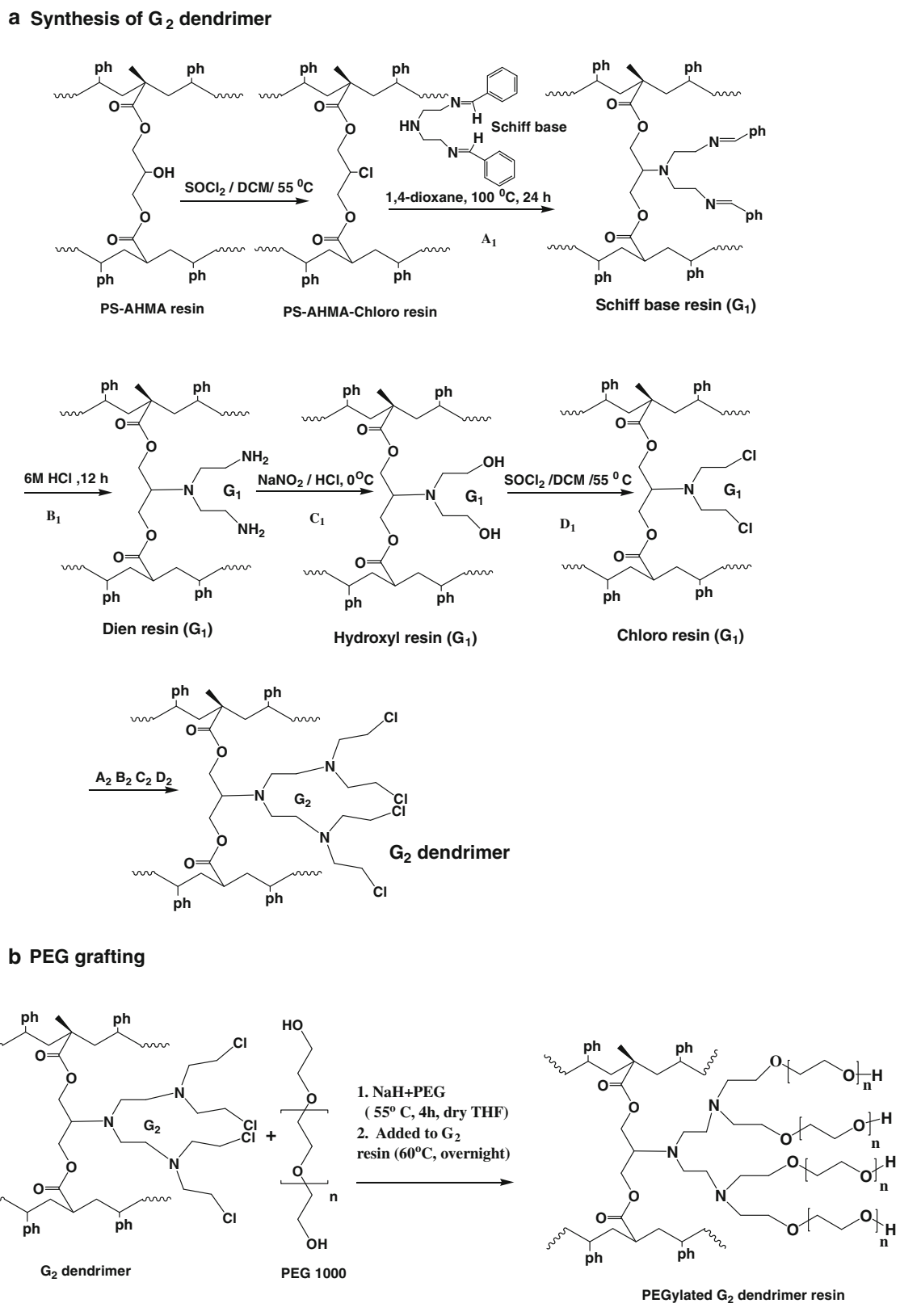
Fig. 1 FTIR spectra of **a** PS-AHMA resin, **b** chlorinated PS-AHMA, **c** Schiff base resin ($\mathbf{G_1}$), **d** amine resin ($\mathbf{G_1}$), **e** hydroxyl resin ($\mathbf{G_1}$), **f** chlorine resin ($\mathbf{G_1}$), **g** PEGylated resin ($\mathbf{G_2}$)

generation by following series of reactions such as Schiff base introduction, acidolytic cleavage, diazotization and thionyl chloride addition. The Schiff base unit bis[2-(Benzylideneamino)ethyl]amine was synthesized by spontaneous exothermic mixing between diethylenetriamine (dien) and benzaldehyde molecules in 1:2 molar ratio and Schiff base formation was verified by ^{1}H NMR analysis. The resulting Schiff base form has structure as shown in Scheme 1a (A_1) in which the ligand can bond to the support exclusively through the imino nitrogen. Poly(*N*,*N*-bisethylamine) dendrimer was synthesized by the selective dehydrohalogenation reaction between chlorine atoms of cross-linker and Schiff base molecules. The direct introduction of dien to cross-linker chlorine will lead to a wide range of products as result of *N*-alkylation at different amine groups in the ligand. Thus, we intended to protect the primary amine groups of the ligand by Schiff base formation prior to immobilization. The different stages of reactions such as Schiff base attachment, acidolytic cleavage, diazotization and thionyl chloride addition (shown as A_1, B_1, C_1 and D_1, respectively), were depicted in reaction Scheme 1a. The FTIR spectrum of Schiff base bound resin shows absorption peak around 1,640 cm^{-1} correspond to –CH = N– linkage of Schiff base units. The grouping of phenyl moieties in the polystyrene backbone will increase the hydrophobicity as well as steric hindrance eventually lead to low-reaction rate and swelling performance. In addition to that, imine linkage bonding between dien and benzaldehyde molecules were not enough stable to survive the various attacks generated by strong acidic reagents. So selective cleavage of imine bond was achieved using 6 M hydrochloric acid resulted in regeneration of resin bound dien molecules which are connected to polystyrene backbone through tertiary amine. After acidic treatment, FTIR absorption peak around 1,640 cm^{-1} disappeared indicating the breakage of imine units and a new peak forms around 3,500 cm^{-1} correspond to regenerated primary amine groups. The loading of amino groups formed were quantitatively estimated by acylation between resin bound free amino groups and Fmoc-Gly-OH to get concordant value of 0.495 mmol/g. The amino loading value indicates the quantitative conversion of cross-linker halogen groups by Schiff base units. The amine resin subjected to diazotization reaction with $NaNO_2$/HCl forms hydroxyl resin ($\mathbf{G_1}$) followed by thionyl chloride reaction lead to first generation chlorine ($\mathbf{G_1}$) dendrimer resin. After diazotization, the absorption peaks correspond to primary amino group's undergone broadening indicating functional alteration to hydroxyl groups and was qualitatively confirmed by negative Kaiser test. The hydroxyl groups formed were estimated by both acetic acid and UV methods, gave concordant value of 0.494 mmol/g. After thionyl chloride treatment, the hydroxyl absorption peak disappeared indicating the conversion to chlorine groups and the amount of chlorine formed were determined by Volhard's estimation method. The estimation was repeated under identical conditions to get the concordant value of 0.493 mmol/g. The various stages of dendron assemblage and functional modifications ($\mathbf{G_1}$) were analyzed by FTIR and results are summarized in (Fig. 1). The identical synthetic paths have been followed (shown as A_2, B_2, C_2 and D_2 in Scheme 1a) to get the $\mathbf{G_2}$ dendrimer having chlorine (–Cl loading = 0.981 mmol/g) as the terminal active groups for PEG-grafting.

In order to confirm the extent of ligand incorporation, $\mathbf{G_1}$ and $\mathbf{G_2}$ amino-dendrimer resins were subjected to CHN analysis. The $-NH_2$ loadings were found out from this data using the expression, % N × 10/14*n*, where % N is the percentage of nitrogen obtained from CHN analysis and *n* is the number of nitrogen atom present in the ligand which is 3. The different functional loading values (mmol/g) estimated and % conversions of chlorine atoms by Schiff base units were summarized in Table 1. The values obtained were in support with results calculated from UV and acetic anhydride measurements and proved as stepwise growth of dendrimeric developed from $\mathbf{G_1}$ to $\mathbf{G_2}$ generations. Chlorinated PS-AHMA resin ($\mathbf{G_0}$) was subjected to Gabriel phthalimide reaction to get amino resin and amino loading was estimated by UV measurement of deprotected Fmoc groups of resin bound Fmoc-Gly-OH as 0.248 mmol/g. The % conversion for $\mathbf{G_1}$ and $\mathbf{G_2}$ resins were calculated from chlorine loading values (obtained from volumetric analysis) and was repeated using amino loading values (obtained from CHN data) using the following expression and the results obtained are summarized in Table 2. For example, % conversion of $\mathbf{G_2}$ can be found out using

$$\%\ \text{Conversion} = \frac{\text{Chlorine capacity of } \mathbf{G_2} - \text{Chlorine capacity of } \mathbf{G_1}}{\text{Chlorine capacity of } \mathbf{G_1}} \times 100$$

Poly(ethylene glycol) (PEG) is chemically inert and robust which can impart hydrophilic character to the



Scheme 1 Synthesis of second generation ($\mathbf{G_2}$) poly(*N*,*N*-bisethylamine) dendrimer on PS-AHMA resin

Table 1 Functional loading values and % conversions calculated from UV and volumetric measurements

Gen.	$-NH_2$ (UV) mmol/g	–OH (UV) mmol/g	–OH (volumetric) mmol/g	–Cl (volumetric) mmol/g	% Conversion
G_0	0.248	0.251	0.251	0.249	–
G_1	0.495	0.494	0.494	0.493	97.99
G_2	0.983	0.982	0.982	0.981	98.96

Table 2 Amino loading values and % conversion calculated from CHN data

Gen.	% N (CHN data)	$-NH_2$ capacity	% Conversion
G_1	2.053	0.489	99.71
G_2	4.086	0.973	98.97

polymer supports and are extensively exploited in different synthetic transformations (Roberts et al. 2002). The crucial objective of grafting PEG onto PS is the combination of a hydrophobic core with hydrophilic PEG chains in the same support. The optimal properties of the PEG are due to the vicinal arrangements of carbon–oxygen bonds throughout the chain, which incite that the PEG assumes helical structures with gauche interactions between the polarized bonds. The amphiphilic nature of PEG makes the resin well-solvated in both polar and non-polar solvents that lead to easy penetration and diffusion of solvents of solid matrices. These optimal properties were achieved by grafting poly(ethylene glycol) (PEG1000) molecules to **G_2** chlorine-terminated dendrimer resin. Instead of grafting PEG-molecules directly to the cross-linker active sites, more exposed and more freely available dendrimeric active sites have been preferred because it helps to accommodate more number of hydrophilic PEG moieties with minimum steric hindrances and considerable loading values. The method of PEG-grafting is shown in Scheme 1b. Initially sodium polyethyleneglycolate was prepared by dissolving weighed quantity of sodium hydride with PEG dissolved in dry-THF under the stream of an inert atmosphere of N_2 gas. The chlorinated dendrimer resins were quantitatively transferred to the reaction mixture for an overnight reaction. The PEGylated dendrimer resins were thoroughly washed with THF, ethanol, methanol, and further solvent extracted with THF. The preliminary evidence of PEG-grafting was observed by increase in mass of resin and was confirmed from FTIR and ^{13}C NMR analysis. The FTIR spectra (Figs. 1, 2) of PEGylated dendrimer resin show an intense broad absorption peak near 3,500 cm^{-1} corresponds to free hydroxyl groups of grafted PEG units. After grafting, a broad and symmetric absorption peak emerged around 1,100 cm^{-1} corresponding to ethereal C–O–C linkage of grafted PEG chains. The formation of PS-AHMA resin, Schiff base incorporation and PEGylation were further confirmed from solid state ^{13}C NMR analysis (Fig. 2). The PS-AHMA support showing absorption peaks around 128 and 154 ppm correspond to aromatic polystyrene and C-3 carbon, respectively, which are present in back-bone. Absorption peaks around 67 and 42 ppm correspond to methylene groups of cross-linker and polystyrene back-bone, respectively. The Schiff base resin showed very small peak around 172 ppm corresponding to imine –CH = N– linkage of introduced Schiff base moieties which were disappeared after acid hydrolysis. After PEG-grafting, an intense absorption peak around 75 ppm appeared corresponds to the C–O–C carbon of PEG units. SEM imaging of beads before and after grafting process also provided sufficient information regarding the extent of drifting of morphological features occurred due to PEGylation. The SEM images of initial as well as grafted resins are shown in Fig. 3a–c. After PEG incorporation, the smooth and homogenous surface of PS-AHMA support becomes rough and inhomogeneous due to the tethering effect of PEG units. The hydroxyl loading of PEG-grafted support was quantitatively estimated by UV measurement and confirmed by acetic anhydride method. Both the experiments were repeated to get concordant value of 0.491 mmol/g.

The swelling nature of polymer supports in polar/non-polar solvents is of paramount importance for the efficient diffusion of reactants into polymer bound active functional sites and for their effective interaction. Instead of dissolving and forming a solution, the cross-linker act as anchor to prevent excessive motion of the polymer chains required to form a solution. Therefore, solvent imbibition ability of chlorine-terminated **G_2** dendrimer and PEGylated resin were measured by syringe method and compared to 4 mol % SAT and commercially available Tenta Gel resins. It is commonly observed that increased cross-linking density severely restricts the solvent uptake ability of polymer supports and has a critical role in deciding the extent of swelling. Therefore, we have studied the relationship between cross-linker densities (2, 4 and 6 mol %) and solvent imbibition abilities of PS-AHMA in commonly using solvents of slightly differing polarities. It was noticed that 4 mol % PS-AHMA showed better swelling and fast achievement of solvent saturation point compared to other two mol percentages and hence preferred for further studies. It was found that grouping of *N*,*N*-bisethylamine dendritic templates not appreciably affect the swelling performance of cross-linked support and it might because of diminutive influence of blended dendritic units on the hydrophobic/hydrophilic nature of the PS-AHMA system. Experimental observation displayed that grafting of

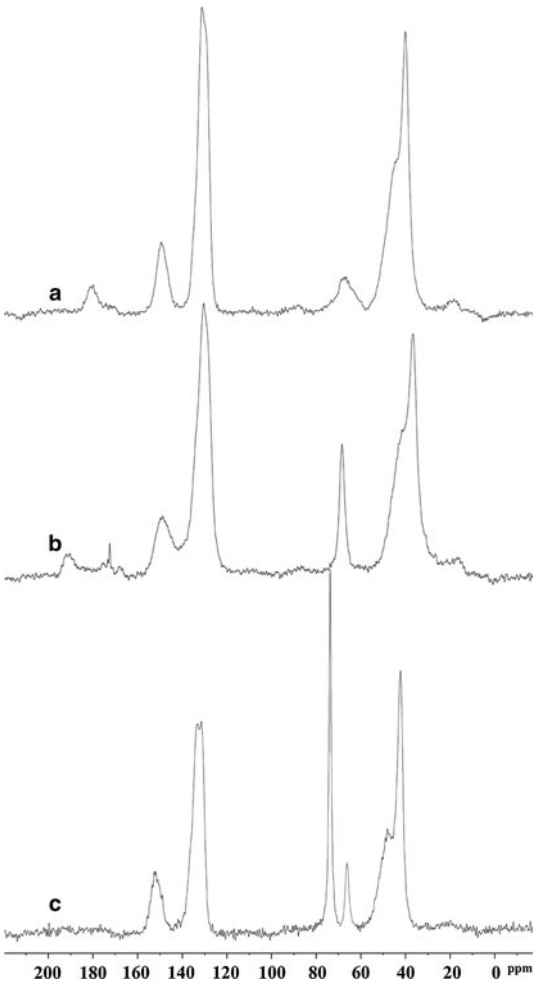


Fig. 2 ^{13}C NMR spectra of **a** PS-AHMA resin, **b** Schiff base resin ($\mathbf{G_1}$), **c** PEGylated resin ($\mathbf{G_2}$)

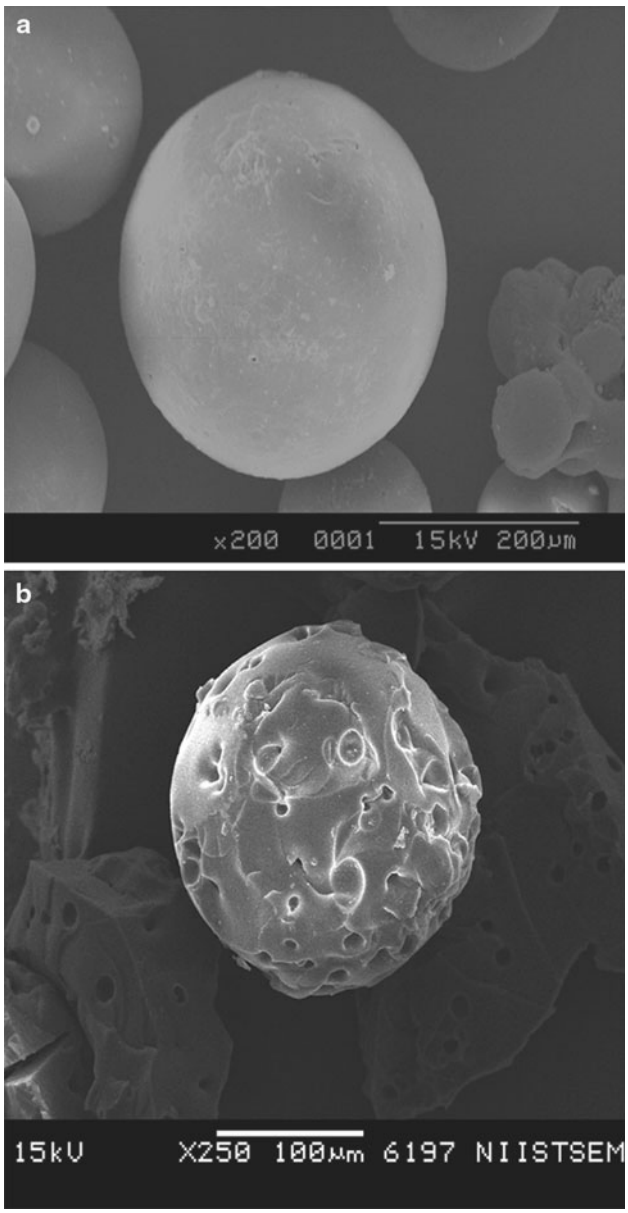


Fig. 3 SEM images of **a** initial PS-AHMA, **b** PEGylated $\mathbf{G_2}$ dendrimer

hydrophilic PEG chains distinctly improves the swelling nature of dendrimer system in both polar and non-polar solvents and the degree of swelling was slightly higher in polar media. These high swelling characteristics are due to the high content of PEG-molecules which readily undergo solvation in polar media and hence better diffusion and penetration of reagent–solvent mixture throughout polymer matrices. The solvent imbibition ability of PEGylated resin was compared to 4 mol % SAT as well as commercially available Tenta Gel resins also and. It was found that PEGylated dendrimer resin allows better diffusion and penetration of solvent molecules in all type of media used for observation and readily accomplish the solvent saturation level, i.e. the saturation stage at which the resin imbibe maximum solvent molecules. These experimental results proved the aptness and efficiency of novel support as an ideal polymer carrier in many reactions such as catalysis and scavenging where fast swelling is essential. These results demonstrated that the characteristic features exhibited by the novel support make it superior than most of commercially available resins and can be effectively utilized for various polymer assisted organic reactions under different synthetic conditions. The swelling results of different cross-linker densities are summarized in Fig. 4a. The comparative swelling results of non-grafted $\mathbf{G_2}$ dendrimer, PEGylated $\mathbf{G_2}$ dendrimer, 4 mol % SAT and Tenta Gel resins are summarized in Fig. 4b.

An inflexible condition for choosing a resin for polymer supported organic synthesis is that the polymer matrix should be chemically stable until the product is separated at the end of the synthesis. Therefore, we have tested the chemical stability of PEGylated resin under various peptide synthetic conditions. The chemical inertness of the resin

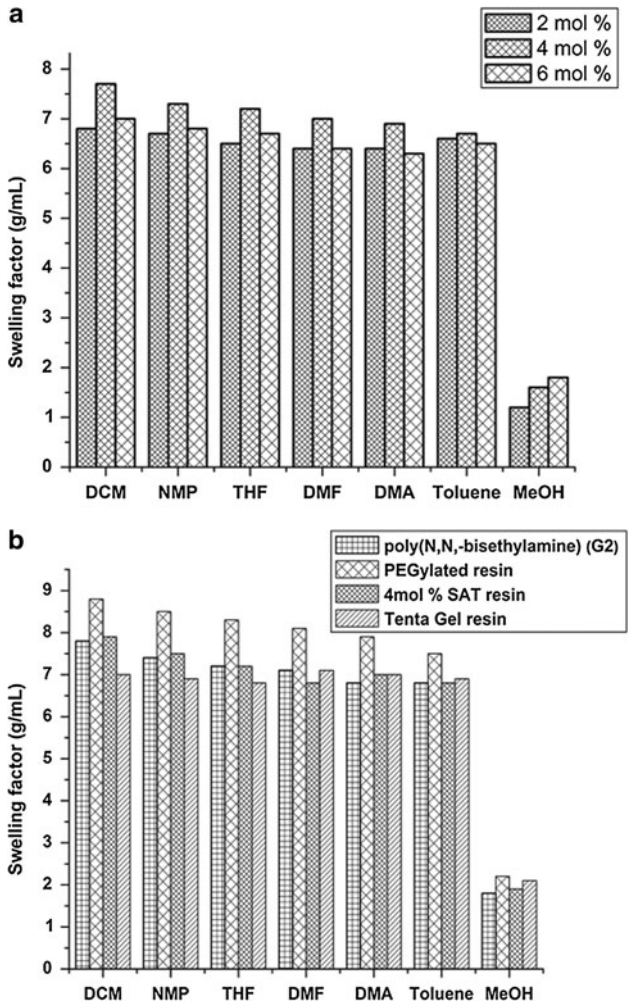


Fig. 4 Swelling comparison of **a** PS-AHMA synthesized in different cross-linking densities of AHMA, **b** PEGylated resin with non-grafted dendrimer, SAT and Tenta Gel resins

was checked by weighing the resin suspended solution before and after treatments as well by recording similar IR spectra. The chemical stability of cross-linker used was already proved under all acidic and basic peptide synthetic conditions (Siyad and Kumar 2012a, b); otherwise some linear polymer will form along with the peptide under cleavage resulting in separation problem. The chemical integrity of PEGylated dendrimeric support was also noticed under different stages of peptide synthesis and observed that poly(*N*,*N*-bisethylamine) dendrimer can withstand the various electrophilic as well as nucleophilic attacks generated by reagents used (Fig. 5). The chemical integrity of PEGylated dendrimer resin toward various reagents/mixture of reagents such as neat TFA, 30 % TFA/DCM, 2 M NH_2OH and 2 M NaOH were checked by exposing resin for 12 h each. As shown in Fig. 5b, c, after TFA treatment, the absorption peak corresponding to hydroxyl functional groups of grafted PEG units become broadened might be due to the protonation of hydroxyl groups of PEG units and tertiary amine groups of dendritic templates. The resulted resin samples were rinsed with alkali solution, washed with water to remove trace amount of alkali and detected with litmus. The sample was dried well before recording the IR spectra and found identical with the original spectrum recorded. The resin-treated solution did not show any significant mass changes proved the chemical stability of support and was further proved by recording identical FTIR spectra. The PEGylated system was suspended in piperidine:DMF (4:1 v/v) mixture, the reagent used for Fmoc removal for 6 h and displayed that the system can withstand in the mixture without undergoing any chemical degradation. When the support was successively shrunk, swelled, and re-shrunk by using different types of solvents, no notable deteriorations of the resin beads were occurred viewed through microscopy. These studies conclude that in contrast to most of other

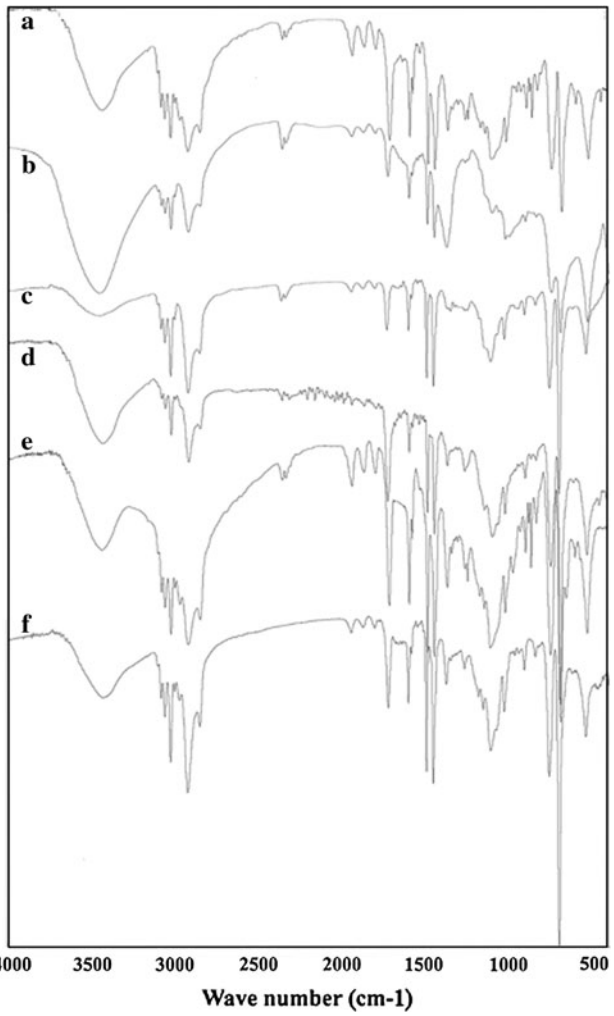


Fig. 5 FTIR spectra of **a** PEGylated resin, **b** 30 % TFA/DCM treated, **c** 100 % TFA treated, **d** 20 % piperidine:DMF(1:4, v/v) treated, **e** 2 M NH_2OH treated, **f** 2 M NH_4OH treated

commercially available resins, which normally fracture during similar treatments, newly developed support was not affected by osmotic shock.

In order to check the applicability of novel dendrimeric support for solid phase peptide synthesis, biologically potent endothelin classes of peptide having amino acid sequence NH_2-Ile–Ile-Trp-Phe-Asn-Thr-Pro-Glu-His-Val-Val-Pro-Tyr-Gly-Leu-Gly-Ser-Pro-Arg-OH was synthesized on HMPB-SAT (500 mg, –OH loading: 0.249 mmol/g) and PEGylated dendrimer resin (500 mg, –OH loading: 0.491 mmol/g) and compared the yield and purity. It has been observed that using PEGylated resin, all the acylation and deprotection reactions were finished with first coupling itself, but for SAT resin, a second coupling was required for completion of acylation with amino acids, such as phenylalanine and tryptophan. A second coupling, however, was given to all acylation for dendrimer support to ensure the reaction completion. Using both supports, the rate deprotection reactions were found to be almost same, finished within 20 min, but 30 min was given to ensure reaction completion. After synthesis, the crude yields of peptides were determined by weighing the peptidyl resins and obtained as 96.3 and 91.4 % for dendrimer resin and SAT resin, respectively. The peptides were liberated from the supports by adding cleavage cocktail for 6 h [TFA:-TIS:H_2O, 95:2.5:2.5, v/v] and subjected to vacuum evaporation, ether precipitation and washings. The peptides were well dried by lyophilization to constant weights and calculated the percentage of yields obtained by comparing the weights of peptidyl resins after synthesis. The peptides were purified by HPLC, powdered by freeze-drying and used for comparison. The percentage yields of the crude as well as purified peptides synthesized using PEGylated and SAT resins were as follows; PEGylated resin: 93.5 % (crude), 89.2 % (corresponding purified) and SAT resin: 86.7 % (crude) and 82.3 % (corresponding purified). The percentage-purified yields of peptides were also calculated using first amino acid substitution values and obtained as 88.5 and 80.9 % for PEGylated and SAT resins, respectively. The purity of crude (shown in Fig. 6a, d) as well as corresponding purified (Fig. 6b, e) peptides obtained from novel dendrimeric and SAT supports were tested by RP-HPLC on a C-18 column. The major peaks have been collected and used for MALDI-TOF analysis and results are summarized in Fig. 6c, d. The yield as well as the purity of peptide synthesized using novel PEGylated dendrimeric support were better than corresponding peptide collected from SAT resin. This might because of high swelling characteristics and high compatibility exhibited by dendrimeric support in reaction medium as well as toward growing peptide chain which allow better diffusion and penetration of solvent–reagent mixture through the course of reaction.

The feasibility of novel support was again checked by synthesizing peptide ET_A receptor antagonist having disulfide bonds connecting between first and fifth positions (Cys^{1-5}) by non-oxidative on resin intramolecular cyclization method (Galande et al. 2005) in which disulfide bond formation was performed prior to cleavage from the support. The peptide having amino acid sequence NH_2-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile–Ile-Trp-OH was synthesized by Fmoc method in which the thiol protecting groups used for Cystein residues were 4-methoxytrityl (Mmt) and tert-Butylthio (S-t-Bu) for first and fifth position, respectively, which are kinetically stable in piperidine/DMF mixture. The cyclization was achieved with 1 % TFA in DCM in the presence of triisopropylsilane (TIS) as the scavenger. The reaction was monitored by measuring the absorbance of 5-nitropyridine-2-thione formed at 386 nm and all the cyclizations were completed within 30 min. The resin was thoroughly washed with DCM and ether and dried under vacuum. The peptide was liberated from the support by suspending resin for 6 h in neat TFA containing triisopropylsilane (TIS), distilled water, and ethanedithiol (EDT) in volume ratio of 94:2.5:2.5:1. The peptide was precipitated as white powder by addition of ice-cold ether, and further washed extensively with cold ether to remove the scavengers. The yield of crude peptide collected was 89.5 %. The HPLC chromatogram of synthetic disulfide-bonded peptide is shown in Fig. 7a. The formation of disulfide linkage was confirmed by collecting the main peak (shown as Fig. 7b) and doing MALDI-TOF analysis in which theoretical mass did match with the experimental result obtained. The yield of pure peptide collected by fractional collection method was 81.2 % and used for MALDI-TOF analysis and the result is summarized in Fig. 7c.

Conclusions

PEGylated poly(*N*,*N*-bisethylamine) dendrimer was synthesized on PS-AHMA resin and evaluated for various stages of solid phase peptide synthesis. Well isolated and less sterically hindered cross-linker active functional sites rather than polystyrene backbone has been chosen for dendron grouping which helps for quantitative incorporation of dendron units and transformation of functional groups. The Schiff base molecules were synthesized by spontaneous exothermic mixing reaction between dien and benzaldehyde units and were bonded to support uniquely through secondary amine by *N*-alkylation reaction. Grafting of amphiphilic PEG-molecules to the chlorine termini of $\mathbf{G_2}$ dendrimer resulted in highly hydrophobic/hydrophilic balanced support having excellent solvation and synthetic performance. The solvent imbibition studies revealed that grafted resin showed admirable swelling

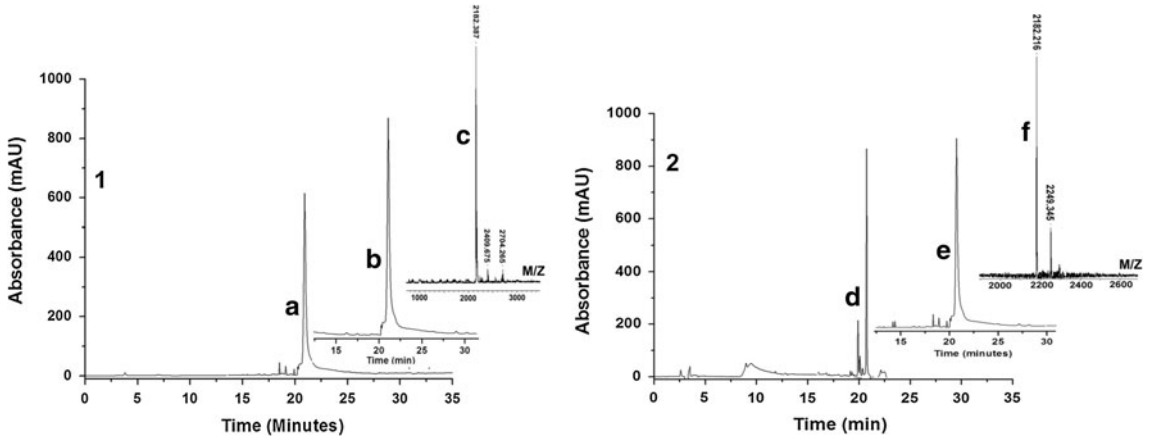


Fig. 6 Endothelin peptides synthesized on **1** PEGylated dendrimer (*a*) Crude HPLC (*b*) purified (c) corresponding MALDI-TOF. **2** SAT resin (*d*) crude HPLC (*e*) purified (*f*) corresponding MALDI-TOF

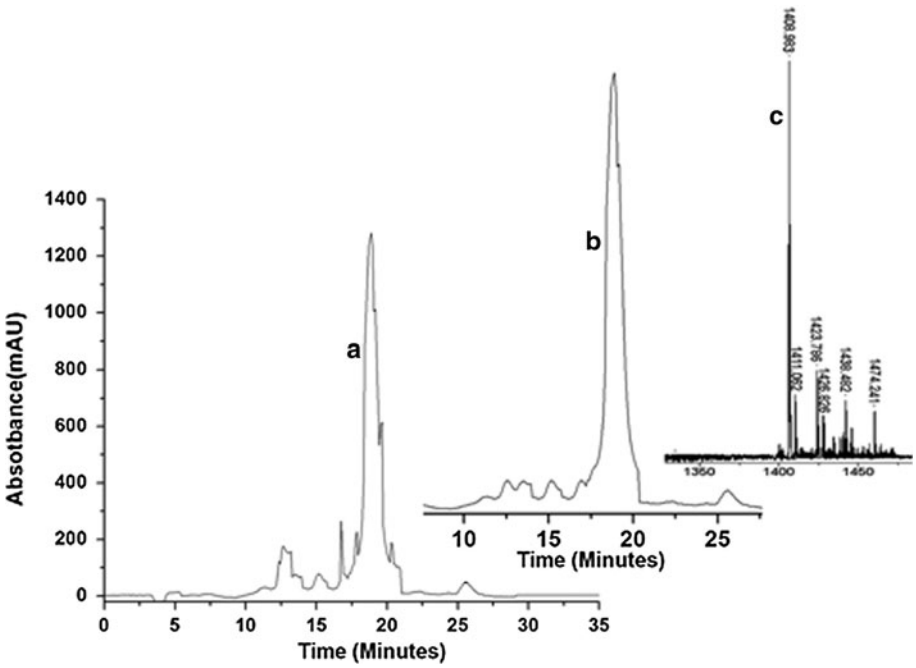


Fig. 7 ET_B receptor antagonist synthesized on PEGylated $\mathbf{G_2}$ dendrimer **a** crude HPLC, **b** purified, **c** MALDI-TOF

characteristics in both polar and non-polar media used for various synthetic conditions. The chemical stability studies demonstrated that PEGylated dendrimer was good enough to resist the various attacks generated by strong acidic and basic reagents.

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