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Supporting Information

A class of linker free amphiphilic PEG grafted polymer support for linear and cyclic peptides

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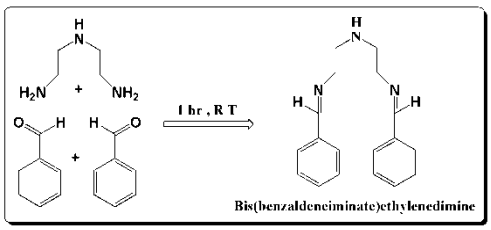
Experimental section

Styrene, DVB, VBC, polyvinyl alcohol (PVA) (Mn~70000), benzoyl peroxide, thionylchloride, diethylenetriamine (dien), benzaldehyde, salicylaldehyde, triethylamine, potassium pthalimide, hydrazine hydrate, Poly(ethylene glycol) (PEG), sodium hydride, diisopropylethylamine (DIEA), triflouroacetic acid (TFA), thioanisol, triisopropylsilane (TIS), 1,2-ethanedithiol (EDT), dimethyl sulfoxide (DMSO), anisol and 1-methylimidazole (MeI) were purchased from Aldrich Chemical Company, USA. All side chain protected F-moc amino acids (L), 2-(1H-benzotriazol-1-yl)1,1,3,3 tetramethyluraniumhexafluoro phosphate (HBTU) and cysteine derivatives were obtained 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. All solvents of HPLC grade were bought from E. Merck (India) and BDH (India). IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The ^{13}C CP-MAS solid state NMR measurements were conducted on a Bruker DSX-300 CP-MAS instrument operating at 75.47 MHz. Optical density (O.D) values were measured with a Shimadzu ultraviolet-visible (UV-VIS) spectrophotometer at 290 nm. SEM pictures were recorded using Hitachi SS 2000 scanning electron microscopy. Mass spectra of peptides were obtained with a Kratos MALDI TOF MS instrument. CHN analysis has been carried out using Elementar Vario EL III. HPLC was performed on a Pharmacia Akta purifier instrument using C-18 reverse phase semi preparative HPLC column. HPLC condition: 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 25 min. and 100 % B in 35 min.

Synthesis of PS-DVB-VBC Resin: Styrene, DVB and VBC were destabilized with 1% NaOH solution (10×10 mL). A four-necked reaction vessel equipped with a thermostat, teflon stirrer, water condenser and N_2 inlet were used for polymerization. 1% solution of PVA (Mw ≈ 70, 000 Da) was prepared by dissolving PVA (1.1 g) in double distilled water (110 mL) and added to the reaction vessel under N_2 atmosphere. For the preparation of 2 mol % cross-linked DVB resin, the monomers, styrene (96 mol %, 10.99 mL), VBC (2 mol%, 0.335 mL) and cross-linker DVB (2 mol%, 0.469 mL) were mixed with toluene (8 mL) and added to the reaction vessel. Different densities of VBC resins were synthesized; 2, 4, 6 and 8 mol % and in all the cases cross-linker density of DVB was kept as 2 mol % and the monomers styrene and VBC were changed accordingly. The resultant solution was stirred at a constant rate of 1200 rpm with a thermostatically regulated mechanical stirrer. The radical initiator benzoyl peroxide (0.5 g) was added to the reaction mixture and kept under continuous flow of N_2 gas. The temperature of the reaction mixture was maintained at 85 °C using a thermo stated oil bath and the reaction was continued for 10 h. The copolymer obtained washed thoroughly with hot water to remove PVA was sohxleted with toluene, dichloromethane, acetone and methanol. Dried in vacuum at 40 °C. The resin collected was 5.8 g.

Synthesis of Bis[2-(Benzaldeneamino)ethyl]amine Schiff Base: Schiff base was prepared by dissolving one molecular equivalent of diethylene triamine (1.061 mL, 10 mmol) with two molecular equivalent of benzaldehyde (2.0312 mL, 20 mmol) dissolved in

absolute ethanol at room temperature. Since the reaction was an exothermic one, an ice cold condition has been maintained for half an hour and brought back the reaction to room temperature before completion. After stirring for 60 min, the volume of solution has been reduced and rotor evaporated to remove the excess ethanol until only oil remained and used for further reaction. The Schiff base formed was confirmed by ^{1}HNMR. ^{1}H-NMR (400 MHz, $CDCl_3$) d = 2.0 (s, 1H,–NH), d = 2.91 (t, 4H, –CH_2, J = 7.1 MHz) d = 3.6 (t, 4H, –CH_2, J = 7.1 MHz), d = 7.5 (m, 4H, J = 7.5 MHz),d = 7.8 (m, 6H, J = 7.5 MHz), d = 8.65 (s, 2H, = CH).



Scheme S1.Synthesis of Schiff Base Dendron.

Schiff Base Introduction to PS-DVB-VBC Resin: PS-DVB-VBC resin (4 g, 0.272 mmol) in 1,4-dioxane (200 cm^3) was stirred for 1 h at room temperature. To this swelled resin, suspension of benzaldehyde Schiff base (1.16 mL, 20 mmol) has been added along with triethylamine (0.5 mL) and heated at100 ^{0}C for 48 h with occasional stirring. Excess ligands act as an acceptor of HCl which was produced by the alkylation. Hydrochloride of the free ligands deposited as yellow crystals during the reaction. The solid materials were filtered off, washed with water to remove the crystalline compound and with dioxane and transferred to a solvent extraction apparatus. The extracted resin was collected, washed with ether and dried at 50 ^{0}C under vacuum. The yield of Schiff base $\mathbf{G_1}$ dendrimer resin ($\mathbf{B_1}$)was 5.28 g.

Schiff Base Cleavage: The Schiff base resin $\mathbf{B_1}$ (5 g) was stirred with 6M HCl (150 mL) at 60 ^{0}C for 24 h. Benzaldehyde was liberated as an oily emulsion during the hydrolysis. Golden yellow polymer beads obtained which are the hydrochloride form of the resin were filtered off, washed with ethanol and ether and dried under vacuum. The resin in the free amino form was obtained by suspending in 0.5 M sodium hydroxide solution (100 mL) followed by thorough washing with water until the solution was neutral. Lyophilised the sample and the free amino groups were qualitatively analyzed by ninhydrin and quantified by UV method by anchoring F-moc-Gly-OH to free amino groups. The free amino loading capacity was 0.134 mmol/g. The yield of $\mathbf{G_1}$ dendrimer amino resin ($\mathbf{C_1}$) obtained was 4.68 g.

Diazotization Reaction: The amino resin $\mathbf{C_1}$ (4 g, 0.536 mmol) taken in a R.B flask was added with 2 M HCl (30 mL) and kept at 0 ^{0}C for 15 min. 5 M sodium nitrite solution (50 mL) was added to the resin in drop wise manner with constant stirring. After the addition, the reaction was allowed to continue in ice cold condition for 1 hr and brought back to room temperature. The diazotized 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 verified using ninhydrin. The negative ninhydrin indicates the conversion of amino to hydroxyl groups. The resin was further treat with ethanol and methanol, then washed with ether and kept in vacuum. The hydroxyl capacity of resin was determined by esterification with Fmoc-Gly-OH using MSNT and Fmoc absorbance measurement at 290 nm. The hydroxyl loading capacity was 0.133 mmol/g. The yield of $\mathbf{G_1}$ dendrimer hydroxyl resin obtained was 4.66 g.

The weighed quantity (100 mg) of dried resin was subjected to acylation with measured amount of acetic anhydride:piperidine mixture (1:4, 3 mL) for 6 h. Ten milliliters of distilled water was added and refluxed for 3 h. The mixture was cooled, filtered and the acetic acid formed was back-titrated with standard (0.1 N) NaOH. A blank titration was also performed. From the titer values, hydroxyl capacity of the resin was calculated and value obtained was 0.133 mmol/g.

Chlorination Reaction: The experimental procedure followed was same as previously reported. Allow the hydroxyl resin $\mathbf{D_1}$ (4 g, 0.532 mmol) to swell in dry DCM for 1 h. To this swelled resin, calculated volume of thionyl chloride (0.381 mL, 10 mmol) has been added in drop wise manner with occasional stirring and kept at 55^0 C for overnight reaction. It was cooled to room temperature and excess thionyl chloride was removed by the slow addition of distilled ethanol with vigorous stirring. The resin was further washed with dichloromethane (5×10 mL), acetone (5×10 mL), ethanol (5×10 mL), methanol (5×10 mL) and ether (5×10 mL) and solvent extracted with ethanol. Kept the resin at 40 ^{0}C in vacuum. The yield of $\mathbf{G_1}$ dendrimer chlorine resin obtained was 3.92 g. The amount of chlorine was estimated by Volhard estimation method and the loading capacity was 0.134 mmol/g.

Development of G_2 and G_3 Dendrimers: Chlorine terminated G_1 dendrimer resin (3.5 g, 0.469 mmol) was further used for the development of G_2 and G_3 dendrimers. We have followed the same reaction pathways such as Schiff base attachment, acidolytic cleavage, diazotization and thionyl chloride reaction for G_2 and G_3 expansion. For the development of higher dendrimer generations, we have taken 1.98 mL (20 mmol excess for G_2 and 3.96 mL (20 mmol excess) for G_3 of Schiff base ligands. The time taken for the replacement chlorine by Schiff base units were 54 and 72 h for G_2 and G_3 generations respectively. The acidolytic cleavage and diazotization of G_2 and G_3 were quantitatively analyzed by Fmoc anchoring, piperidine induced F-moc release and spectrophotometric analysis of flurenylmethyl-piperidine adduct. Thionyl chloride addition followed by Volhard's quantification gave chlorine loading values of 0.264 and 0.516 mmol/g for G_2 and G_3 respectively. The % of nitrogen obtained from CHN analysis, amino loading values calculated and % conversions for G_1, G_2 and G_3 dendrimer resins are summarized in Table 1.

Table S1. Amino Capacities and % Conversions Calculated From CHN

Gen.	% N (CHN data)	-NH_2 Capacity (mmol/g)	$\left[\frac{\%\ N}{14n} X\ 10\right]$	% conversion
G_1	0.559	0.133		98.60
G_2	1.093	0.260		99.71
G_3	2.163	0.515		98.41

PEGylation (PEG_{400}& PEG_{1500}) to G_3 (E_3)Dendrimer Resin: Sodium polyethyleneglycolate solution was prepared by adding sodium hydride (119.4 mg, 4.98 mmol) to weighed amount of polyethylene glycol (1.99 g, 4.98 mmol for PEG_{400} and 7.47 g, 4.98 mmol for PEG_{1500}) dissolved in dry THF till a green color persisted and stirred for 15 minutes; weighed amount of chlorinated resin (1 g, 0.516 mmol), one gram each for PEG_{400} and PEG_{1500}, and excess amount of PEG (0.5 g each for PEG_{400} and PEG_{1500}) were added to this solution and heated under reflux for 12 h in an atmosphere of N_2. The polymer beads washed thoroughly with dilute HCl (5×5 mL), ethanol (5×15 mL), methanol (5×15 mL), dichloromethane (5×15 mL), acetone (5×15 mL) and ether (5×5 mL) were further solvent extracted with THF. The resins were then washed with ether and dried at room temperature in vacuum. The yields of the PEGylated resins obtained were 1.431g and 1.978 g for PEG_{400} and PEG_{1500} respectively. Hydroxyl loading values of PEGylated dendrimeric supports were determined by Fmoc-Gly-OH/MSNT/MeI and acetic anhydride methods and obtained as 0.203 and 0.326 mmol/g for PEG_{1500} and PEG_{400} respectively. The discrepancy in –OH capacity and mass obtained might be due to chain formation effect in which polymeric PEG chains make cyclic chain between vicinally placed –Cl groups of dendrimer chains. So all the grafted PEG chains will not be available as free –OH groups for further organic synthesis.

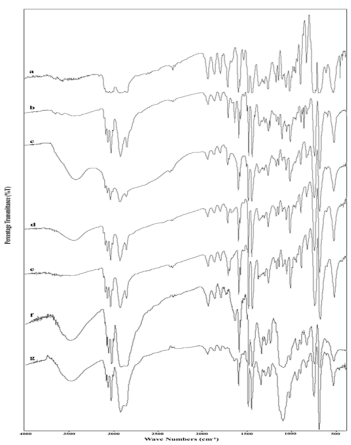
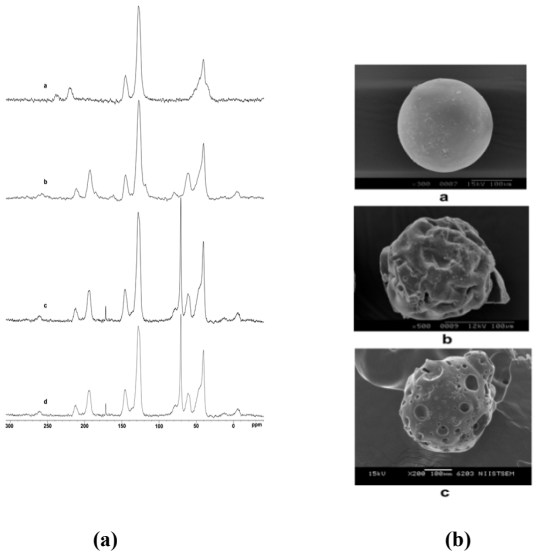


Figure S1. FTIR spectra of (a) PS-DVB-VBC resin (b) Schiff base resin (G_1) (c) Amino ethyl resin (G_1) (d) Hydroxy ethyl resin (G_1) (e) PEG_{400} grafted G_3 resin (f) PEG_{1500} grafted G_3 resin.



(a) **(b)**

Figure S2 a&b. (a) ^{13}C NMR spectra of (a) PS-DVB-VBC resin (b) Schiff base resin (**G_1**) (c) PEG_{400} grafted (**G_3**) (d) PEG_{1500} grafted (**G_3**). (**b**) SEM images of (a) PS-DVB-VBC (b) PEG_{400} grafted (**G_3**) (c) PEG_{1500} grafted (**G_3**).

Chemical Stability Studies: The chemical inertness of PEGylated dendrimeric support under different peptide synthetic conditions were carried out using reagents such as 100 % TFA (10 mL), 20 % piperidine in DMF (10 mL), 2 M aqueous NaOH (10 mL), 2 M NH_2OH in aqueous methanol (10 mL) and liquor ammonia (10 mL). The resin samples (100 mg of each) were separately stirred with above reagents. After 48 h the resin samples were filtered, washed thoroughly with ethanol (3×50 mL), water (3 ×50 mL), acetone (3×50 mL), dichloromethane (3×50 mL), dioxane (3×50 mL) and ether (3×50 mL). Dried and weighed (100 mg); IR (KBr) spectra of treated samples were recorded and compared to original.

Solvent Uptake Studies: Solvation ability of PEGylated supports were (1 g each) determined by syringe method in solvents of differing polarities. In a standard protocol, the PEG_{400}& PEG_{1500} grafted resins (1 g each) were placed in a syringe fixed with a teflon filter at the bottom. The solvent was sucked into the syringe and after 3 h; excess solvent was removed by applying force on the piston. The extent of swelling of the resin in each solvent was determined from the volume of the resin placed after the solvent incubation and the amount of dry resin. The experiment was repeated to ensure reproducible values. The un-grafted **G_3** generation dendrimer resin was also used to evaluate the efficiency of PEG grafting and compared to the commercially available Merrifield resin.

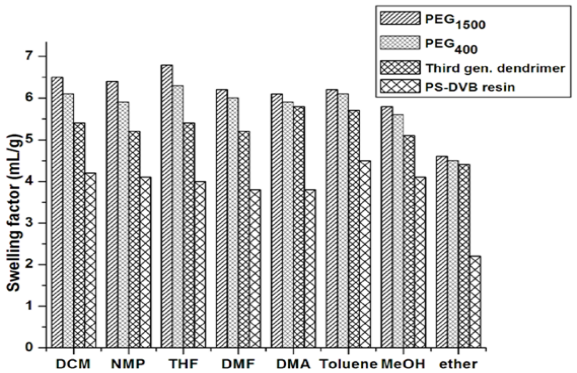


Figure S3. Swelling comparison of PEGylated dendrimers with Merrifield and hydroxy ethyl dendrimer (**G_3**) resins.

Peptide Synthesis: Standard Fmoc strategy has been followed for entire peptide synthesis. Since PEG will act as a spacer which connects the first amino acid and hydroxyl end of resin, Fmoc-Trp(Boc)-OH was initially anchored to the hydroxyl groups of PEG grafted resins using MSNT and 1-methyl imidazole (MeI) without preferring any linker in dry DCM. Fmoc protection was removed by using 20% piperidine in DMF. In a typical coupling step, HBTU and HOBt were added to the Fmoc amino acid dissolved in DMF in presence of DIEA. The mixture was stirred and added to the resin swollen in DMF, and the reaction was allowed to continue for 60 min. The extent of coupling was monitored by Kaiser test. After each coupling and deprotection steps, the resin was thoroughly washed with DMF (5 × 50 mL). When desired sequence of amino acids were attached to the resins, they were washed with DMF (5× 50 mL), DCM (5 × 50 mL), and ether and dried under vacuum. The yield of peptide was calculated by comparing the weight of the peptidyl resin and the amount of peptide obtained just after cleavage and purification.

Linear Peptide Synthesis (Peptide 1): Synthesis of ET_B receptor antagonist having amino acid sequence H_2N-Ala-Ser-Ala-Ser-Ser-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp-OH was carried out by weighing 300 mg each of PEG_{1500} and PEG_{400} grafted polymer supports (-OH loading values were 0.061 and 0.098 mmol for PEG_{400} and PEG_{1500} resins respectively) under identical synthetic conditions and compared. The weighed supports were quantitatively transferred to two silanized 15 mL glass peptide synthesizer containing a sintered filter on one side and a receiving adapter fitted with a calcium chloride guard tube on the other. The first C-terminal attachment was carried out by MSNT coupling under dry conditions using mixture of Fmoc-Trp(Boc)-OH/MSNT/MeI (0.069 g: 0.033 g: 8 μL, 2:2:1.5 equiv excess) in the following ratio of 1:1:0.75 in dry DCM. Complete incorporation of first amino acid was quantitatively verified by spectrophotometric measurement of the adduct dibenzofulvene-piperidine formed. Removal of Fmoc group was achieved using 20% piperidine in DMF (2 mL) and free amino groups were qualitatively checked by Kaiser test. Further coupling reactions were carried out using 2 equiv excess of Fmoc amino acids, HBTU(0.049 g, 2 equiv excess), HOBt (0.018 g, 2 equiv excess), and DIEA (22 μL, 2 equiv excess) in DMF for both PEG_{400} and PEG_{1500} grafted solid supports. The peptidyl supports were suspended in a mixture of cleavage cocktail having TFA (4.70 mL), ethanedithiol (0.125 mL), triisopropylsilane (0.05 mL) and double distilled water (0.125 mL) and kept at room temperature for 6 h. The resins were filtered off, washed with neat TFA (two times) and rinsed with DCM (2×3 mL) and vacuum evaporated to obtain a thick oily residue. The peptides were precipitated as white powder by addition of ice cold ether followed by washing thoroughly with cold ether (10×10 mL) to remove the scavengers. Dried by lyophilization and used for HPLC and MALDI-TOF analysis.

Air Oxidation Method (Peptide 2): ET_B receptor antagonist having amino acid sequence H_2N-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp-OH was synthesized on weighed quantity of (200 mg) PEG_{400} grafted support (0.061 mmol) which was first allowed to swell in dry DCM (3 mL) for 30 min. Drained off the solvent, dissolved MSNT/F-moc-Trp(Boc)-OH/1-MeI in dry DCM in the ratio 1:1:0.75 and transferred to the resin and allowed to stand for one hour with occasional stirring. Deprotection followed by coupling of each amino acid was continued till the desired length of peptide is synthesized. Detach the peptide from the support using cocktail which comprised of TFA (4.70 mL), ethanedithiol (0.125 mL), double distilled water (0.125 mL), triisopropylsilane (0.05 mL) and allowed to stand for 5 h. The combined filtrate was concentrated under reduced pressure and the peptide was precipitated by pouring cold ether. In the course of washing the peptide, the exposure it to atmospheric air was minimized by using septum-stoppard centrifugal tubes to resist the disulfide bond formation. Dried the peptide by lyophilization and for disulfide bond was formed by air oxidation process. Dissolve the peptide in 0.1 M solution of deaerated ammonium bicarbonate solution (2.5 mg/mL). Kept the mixture to stand open to atm with occasional stirring until the disulfide formation were completed. The reaction was monitored by Ellman test in which 5,5'-Dithiobis(2-nitrobenzoic acid) reacts quantitatively with aliphatic sulfhydryl groups to generate yellow anion to follow the progress of air oxidation by measuring the UV absorbance at 410 nm. Dried the peptide by lyophilization and used for HPLC analysis.

TFA/DMSO/Anisole Method (Peptide 2): The peptide 2 was synthesized on PEG_{1500} grafted support (200 mg, 0.065 mmol), cleaved and was precipitated by adding chilled ether. The precipitate was washed with ether until the scavengers were removed and dried by lyophilization. Crude peptide (10 mg) was dissolved in 10 mL TFA: DMSO: Anisole (97.9:2:0.1 v/v) mixture in a 50 mL round bottom flask and the mixture was allowed to stand for 1 h at room temperature with occasional stirring. The excess TFA was removed in vacuum and the peptide formed was precipitated by adding ice cold ether. The peptide was dried by lyophilization and was checked for the disulfide bond formation by HPLC and MALDI analysis.

Combination of Air and TFA/DMSO/Anisole Oxidation Method (Peptide 3): Accurately weighed (200 mg) PEG_{1500} grafted F-moc-Trp(Boc)-resin (-NH_2 loading, 0.065 mmol) was used for synthesizing 21mer peptide having amino acid sequence H_2N-Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp-OH. After the desired sequence of amino acids has been incorporated, the peptidyl resin subjected to cleavage using the cocktail comprising of TFA (4.70 mL), was double distilled water (0.125 mL), ethanedithiol (0.125 mL) and triisopropylsilane (0.05 mL) and allowed to stand for 5 h at room temperature. The excess TFA was removed under reduced pressure and peptide was precipitated by adding chilled ether. The scavengers were removed by washing with ether repeatedly in air tight septum-stoppered centrifugal tubes and finally dried by lyophilization. Dissolve the peptide (10 mg) in 0.1 M solution of deaerated ammonium bicarbonate solution (2 mL) and allowed the peptide solution to stand open to atmosphere with occasional stirring until the disulfide formation was completed. The reaction was monitored by Ellman test at regular intervals in which 5,5'-Dithiobis(2-nitrobenzoic acid) reacts quantitatively with aliphatic sulfhydryl groups to generate yellow anion and progress of air oxidation was measured by UV absorbance at 410 nm. The pure peptide formed was collected by fractional collection method and lyophilised the sample and used for second disulfide bond formation. 5 mg of crude peptide was dissolved in 5 mL of TFA: DMSO: Anisole (97.9:2:0.1, v/v) mixture in a 50 mL round bottom flask and the mixture was allowed to stirr for 1 h at room temperature with occasional swirling. The excess TFA was then removed under reduced pressure and the peptide was precipitated by adding chilled ether. Dried the peptide by lyophilization and HPLC analysis was done.