Solid-Phase Peptide Synthesis of Endothelin Receptor Antagonists on Novel Flexible, Styrene−Acryloyloxyhydroxypropyl Methacrylate−Tripropyleneglycol Diacrylate [SAT] Resin

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Novel cross-linked polymeric support by the copolymerization of styrene and 3-(acryloyloxy)-2-hydroxypropyl methacrylate with Tri(propyleneglycol) diacryalte (SAT) for solid-phase peptide synthesis is presented here. The synthesis of SAT is based on the cross-linking of 3-(acryloyloxy)-2-hydroxypropyl methacrylate with styrene by free-radical suspension polymerization, consisting of an ester and a secondary hydroxyl group. An additional cross-linker tri(propyleneglycol) diacryalte provides a hydrophilic environment throughout the resin, which will enhance the physicochemical properties of the resin toward organic synthesis. The resins were synthesized in various cross-linking densities to check the swelling property, mechanical stability, and functional loading capacity. The resin was characterized by the IR, ^{13}C NMR, and SEM techniques. The extent of swelling properties of the polymer of different cross-linking densities were studied and compared with Merrifield resin and TentaGel. To demonstrate the efficiency of SAT support was proved by synthesizing the challenging peptide sequence of acyl carrier protein (ACP) and compared with commercially available Merrifield resin. It was further tested by synthesizing endothelial receptor antagonist peptides using SAT resin and compared with commercially available TentaGel resin. The standard Fmoc strategy was adopted for peptide synthesis and was characterized by MALDI-TOF MS and analyzed the purity of peptides by HPLC.

Introduction

The technique solid phase peptide synthesis (SPPS) is based extensively on the pioneering work of Merrifield.[1] SPPS naturally lends itself to the production of peptides because of the limited range of synthetic transformations that are required for synthesis, and each of the key reactions has been optimized to allow the production of peptides of sizable length and high yield.[2−7] The polystyrene resins were used extensively for many years, but there was a growing realization that the nature of the local environment around the growing molecular chain had a significant effect on the rate and extent of reaction.[8] Polystyrene is completely hydrophobic in nature, whereas the growing peptide chain is much more hydrophilic and this difference induces a chain-folding effect in which the peptide satisfies its own hydrogen-bond requirements rather than being solvated. This can severely limit the synthetic access to the exposed end of growing chains. The chemical nature and topographical structure of the polymer matrix are important parameters determining the physicochemical properties required for efficient peptide synthesis. An alternative route to increase the yield of stepwise chain assembly focused on improving the polarity of the original PS-DVB resin to afford greater accessibility of the growing peptide chain to acylation and deprotection. Grafting of the polystyrene core with polar linear polymers, such as poly(ethylene glycol) (PEG), has proved that it improves the swelling of the resin in polar solvents.[9−13] A number of different strategies have been employed to prepare alternative supports that avoid the limitations of PS-DVB.[14−19] To circumvent the inherent problems associated with PS-DVB resins, new cross-linkers have been designed both to increase the flexibility of the polymer backbone to allow better diffusion through the matrix and also to impart a variety of solvent like properties of the resin.

One important step that makes the polymer suitable for polypeptide synthesis is the initial functionalization step. Generally in styrene based resins this can be achieved by various techniques. Functionalization is usually carried out either in the para position, or substitution takes place at ortho position of the phenyl ring of the polystyrene. Another possibility is the functionalization of the neighboring phenyl groups of the polystyrene and peptide synthesis under these conditions can affect the purity of the final peptide. Chloromethylation using chloromethyl methyl ether can sometimes result in the formation of methylene bridges between the phenyl rings that increases the rigidity of the resin. To avoid all these problems of styrene-based resins, a new cross-linked system styrene−acryloyloxyhydroxypropyl methacrylate−tripropyleneglycol diacrylate (SAT) resin having single functional site in cross-linker instead of a multifunctional site in styrene backbone is introduced. It gives high

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swelling property to the entire system because of its semihydrophilic nature causing high yield and purity. The important property associated with this system is that functional sites are specific and the new polymeric support designed for SPPS is devoid of the problems mentioned above. The unique advantage of SAT-resin compared with other polymeric systems is that the coupling can be started from the cross-linker after functional modification. The aim of the present work is to design a novel polymer system and a new technique for rectifying the drawbacks in solid-phase peptide synthesis to obtain peptide with high yield and purity. The efficiency of the SAT-resin is proved by synthesizing the difficult sequence of acyl protein carrier fragment and compared with commercially available Merrifield resin. The present resin is also used for synthesizing the potent cardiovascular drug endothelin receptor antagonists.

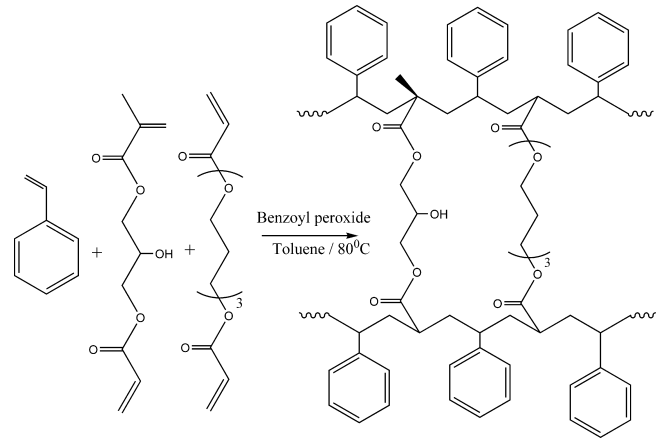
Results and Discussion

SPPS enables us to prepare organic compounds especially peptides and proteins easily and conveniently and, hence, to build up combinatorial libraries for high-throughput screening. The success of SPPS depends on the accessibility of resin bound functional groups to reagents and solvents. In solid-phase resins, the utmost importance is given to the physical property of swelling. Clearly, the ability of a cross-linked polymer to take up solvents is of supreme criteria. The polymer with optimum hydrophobic/hydrophilic balance that swells uniformly both in polar and nonpolar solvents is considered to be the ideal support for SPPS.

The SAT polymer was prepared by copolymerizing hydrophobic styrene monomer and hydrophilic cross-linkers like 3-(acryloyloxy)-2-hydroxypropyl methacrylate (AHMA) and tri(propyleneglycol) diacrylate (TPGDA) using benzoyl peroxide as free radical initiator. SAT polymer support shows comparable mechanical stability with PS-DVB resin and the hydrophilic nature of cross-linker assists the resin to be physicochemical compatible with the resin bound biopolymers, such as peptides. In solid-phase organic synthesis, suspension polymerization is the preferred mode for resin synthesis. Suspension polymerization comprises a two-phase system, where the monomer and porogen (toluene) exist in one phase, suspended in an excess of nonsolvent water in other phase. The polymerization was conducted by using an aqueous solution of 1% PVA ($M_w \approx 75\,000$ Da). The solution was deoxygenated by continuous flow of N_2 gas until the solution became clear. Mechanical stirring of this solution at 1200 rpm results in the formation of uniform droplets of the dispersed organic phase in the dispersion medium. The stabilizer poly(vinyl alcohol) solution prevents the respective droplets from coagulation. SAT resins with different cross-linking densities (2, 4, 6, and 8 mol %) were synthesized (Scheme 1).

The radical initiator, solubilized in the organic phase, promotes the free radical polymerization induced by high temperature. The chain initiation, chain propagation, and chain termination reactions proceed in each droplet. The choice of the diluents, amount of the diluents, aqueous phase to organic phase ratio, size and the shape of the reaction vessel, and stirring speed will affect the polymerization and

Scheme 1. Synthesis of SAT Polymer Support



the bead size of the polymer. Increasing the aqueous phase-to-organic phase ratio results in a polymer with a smaller bead (<100 mesh) size being obtained. This can also be achieved by increasing the stirring speed. It is possible to target a specific range of bead size by carefully adjusting the type of the diluents, aqueous phase-to-organic phase ratio, and stirring speed.

The polymer obtained in bead shape of 100−200 mesh size was used for peptide synthesis and was characterized by scanning electron microscope (Figure 1a and b). The integrity of polymer was confirmed by solid-state ^{13}C NMR spectroscopy with an intense peak at 130.88 ppm (aromatic polystyrene carbons) and a peak at 148.83 ppm for the C-3 carbon of the styrene. The peak at 65.42 ppm (methylene carbon of the cross-linker), which contains secondary hydroxyl groups and 43 ppm correspond to the backbone methylene carbon of the polymer (Figure 2). For IR characterizations, the resin was powdered and palletized with KBr. Initial SAT resin shows sharp bands at 1718 cm^{-1} corresponding to the ester carbonyl of the cross-linkers, 1122 cm^{-1} corresponding to C−O stretching, and 3464 cm^{-1} corresponding to the hydroxyl stretching vibration, in addition to the usual peaks of polystyrene. After chlorination, the peak at 3464 cm^{-1} disappears completely indicating the conversion of hydroxyl to chlorine. The amino SAT resin was prepared from the chlorinated resin by treating potassium phthalimide followed by hydrazinolysis. The amino SAT resin gives an intense blue color with ninhydrin and gives a characteristic IR (KBr) absorption at 3420 cm^{-1} indicating N−H vibrations (Figure 3a−c).

Swelling Comparison of SAT Resin with the Commercially Available PS-DVB and TentaGel. The swelling characteristics of SAT-resin with different solvents were compared with commercially available PS-DVB and TentaGel resins (Figure 4a and b). The swelling studies were conducted using a syringe fitted with sintered Teflon filter. The degree of swelling in various solvents is normalized by calculating the weight of the solvent per gram of the resin. The 2% SAT and TentaGel resin shows a high degree of swelling character in a broad range of polar and nonpolar solvents compared to that of commercially available PS-DVB. The swelling of the new resin is almost twice compared to PS-DVB resin in all solvents.

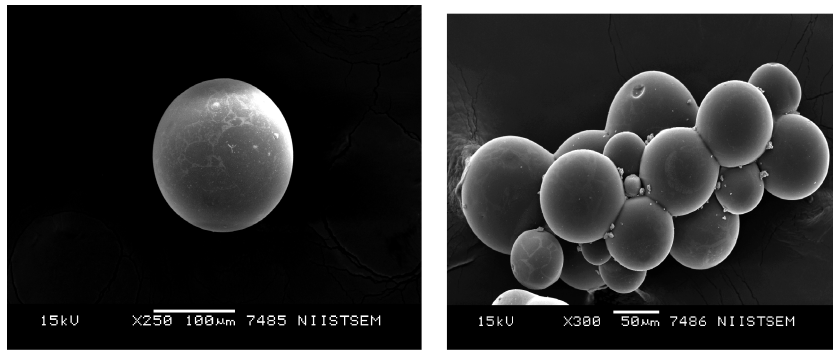


Figure 1. (a and b) Scanning electron micrographs of SAT spherical beaded resin prepared by suspension polymerization.

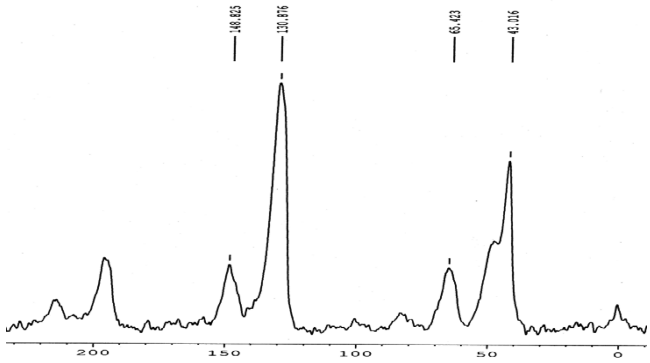


Figure 2. ^{13}C CP-MAS NMR spectrum of SAT resin.

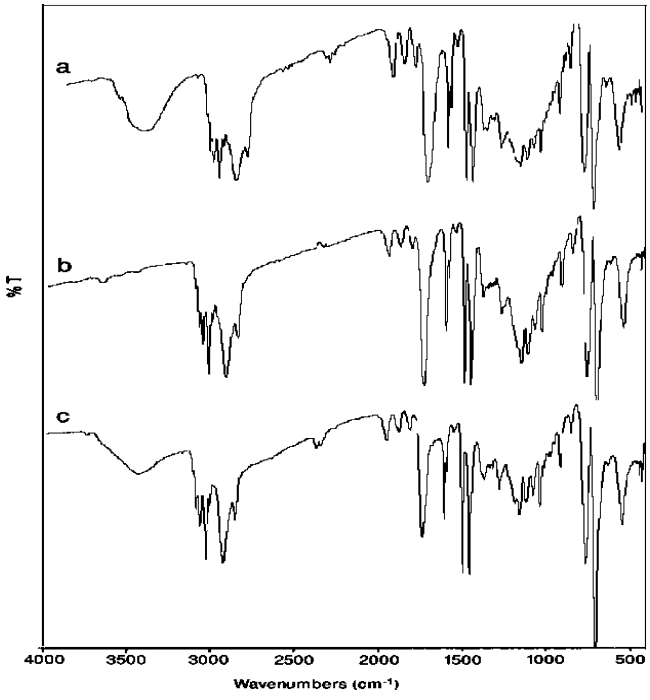


Figure 3. IR spectra of (a) SAT resin, (b) chlorinated resin, and (c) aminated resin.

Solvation is an indispensable condition favoring the organic chemical reactions in gel-phase. Swelling of the polymer allows the effective diffusion of solution phase reagents to polymer bound functional groups. For an effective SPPS, the accessibility of the N-terminal moiety of the growing resin bound peptide chain to reagents and solvents is very important. The reactive functional group in the resin will have maximum accessibility toward the reactants, only

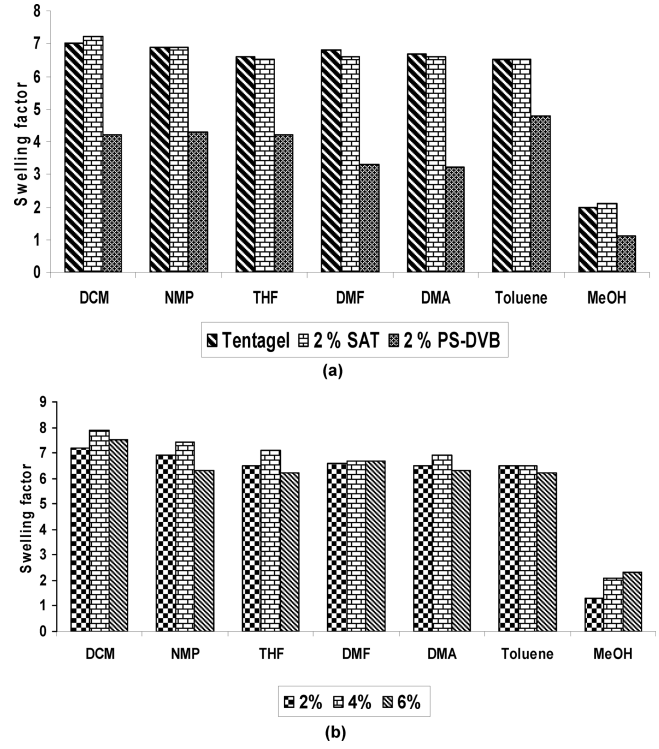


Figure 4. (a) Swelling comparison of 2 mol % SAT-resin with PS-DVB and TentaGel resin. (b) Swelling characteristics of different cross-linking densities of SAT resin.

when polymer matrix swells extensively in the solvating medium. The swelling behavior of SAT resin in organic solvents is found to decrease with increase in the amount of cross-linker in the resin, where, as in polar organic solvent, the trend in swelling behavior is exactly opposite even though there exists a decrease in swelling behavior in higher cross-linked resins. Swelling nature of various cross-linking densities (2, 4, and 6%) was studied. When the swelling nature of the resin in all solvents is compared, the 4 mol % cross-linked system shows higher swelling, and it was used for the synthesis of endothelin class of peptides.

Chemical Stability of SAT Resin. A stringent condition for choosing an ideal solid support for SPPS is that the resin should be chemically stable until the peptide is separated at the end of the synthesis. So the chemical inertness of the support is tested by treating with neat TFA at 30 °C. Apparently, the cross-linked resin is stable enough to withstand the TFA activity. The TFA solution did not show

any mass change after the treatment, which provides primary indication of nondegradability of solid support. The stability of resin was further confirmed from the identical IR spectra of the TFA treated and untreated resin. The support showed comparable physical, chemical, and mechanical properties to that of PS-DVB support. The stability of resin was tested by various peptide synthetic conditions; 20% piperidine in DMF (reagent used for Fmoc deprotection) for 48 h does not show any change in its IR spectrum. The resin does not show any considerable change in IR spectrum even after treatment with aqueous NH_2OH and NH_4OH. After 48 h treatment with 30% TFA in DCM (the reagent used for Boc-deprotection), the resin shows no observable change in IR spectrum, which proves that the resin has enough stability for peptide synthetic conditions (Supporting Information Figure 1).

Acyl Carrier Protein Fragment Synthesis on SAT and Merrifield Resin. To evaluate the applications of the novel user-friendly SAT support for solid-phase peptide synthesis, 2 mol % SAT resin (functional loading 0.126 mmol/g) was synthesized and used for comparative study of a classic difficult sequence, acyl carrier protein, ACP^{65-74}, having amino acid sequence $V^{65}Q^{66}A^{67}A^{68}I^{69}D^{70}Y^{71}I^{72}N^{73}G^{74}$; 2 mol % SAT and PS-DVB resins were attached with HMPA linker and used for the synthesis. In the case of SAT resin, it is found that for every amino acid only 2 mmol excess of the reagent is required. However for PS-DVB support, the entire reagent could be driven to completion with 3 mmol excess of reagents, and it also took a prolonged time period for completion. The reactions, being diffusion controlled, highly depend on the solvation property of the polymer matrix. In PS-DVB because of the hydrophobic core, the reactive sites are not fully exposed; hence, a prolonged reaction time and an excess of reagents are necessary for complete conversion. But in the case of SAT resin, because of the presence of hydrophilic hydroxyl cross-linker and flexible tripropyleneglycol cross-linker, the coupling reaction takes place faster than in PS-DVB resin. After the entire synthesis, the peptide was cleaved from the support using the cleavage cocktail, comprising TFA and cation scavengers. It was finally precipitated using ice-cold diethyl ether. The peptide was washed thoroughly with ether to remove the scavengers. After synthesis, the peptides were removed from their corresponding resins under the same cleavage conditions. The yields of peptide synthesized on SAT resin were observed as 75% (crude yield after synthesis), 69% (crude yield after resin cleavage), and 64% (yield after HPLC purification). But in Merrifield resin, the yields were 52% (crude yield after synthesis), 45% (crude yield after resin cleavage), and 38% (yield after HPLC purification). The purities of acyl carrier protein fragments obtained from the SAT and Merrifield resins were tested by HPLC using C-18 column. The HPLC profiles of crude peptides obtained in PS-DVB and SAT are shown in Supporting Information Figure 2a and b. The targeted peptide synthesized by PS-DVB was indicated by an arrow in Supporting Information Figure 2a. These results indicate that purity of peptide synthesized using SAT resins is higher than that of Merrifield resin. These high reaction efficiencies may be the result of the long, flexible, hydrophilic, and uniformly distributed cross-linkers introduced in the resin matrix. The crude peptides obtained from PS-DVB and SAT were purified and analyzed by HPLC (Supporting Information Figure 2c and d). The mass of purified peptides synthesized on PS-DVB and SAT were confirmed by MALDI-TOF (Supporting Information Figure 3a and b). The comparative analysis shows that the SAT resin can be used as a better solid support for peptide synthesis than Merrifield resin.

Endothelin Peptide Synthesis. The endothelin family of peptides is very potent endogenous vasoconstrictor and precursor agent, secreted by various cells and tissues in the human body. Two endothelin receptor subtypes have so far been cloned in mammalian species, ET_A and ET_B. Since their discovery in 1988, the endothelin species have been the subject of intense research on their physiological function and potential pathophysiological role in various disease states. The following endothelin receptor antagonists peptides were synthesized using 4 mol % SAT resin and PEG grafted TentaGel resin: peptide1, Ac-His-Leu-Asp-Ile-Ile-Trp-OH; peptide 2, H_2N-D-Trp-Leu-Asp-Ile-Ile-Trp-OH. To prove the efficiency of the resin, we synthesized long peptide endothelin receptor anatagonist (peptide 3) having amino acid sequence H_2N-Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp-OH. The C-terminal tryptophan was attached to the resin via an ester bond using MSNT in solvent dry DCM. The extent of esterification was measured from the UV absorbance of the adduct of dibenzofulvene and piperidine. The peptides were liberated from the supports using neat TFA in the presence of scavengers. The yield and purity of peptides synthesized on SAT resin are almost same as that of TentaGel resin, which proves the efficiency of newly developed support. The time-dependent Fmoc deprotection was also followed with SAT and Merrifield resin of same hydroxyl capacity (0.249 mmol/g) using Fmoc tryptophan as shown in (Figure 5a and b). The rate of deprotection found to be much faster in SAT than in Merrifield resin because the hydrophilic character of the cross-linker present in SAT favors the easy penetration of the reagent to the polymer core.

The yield of the crude and purified peptides synthesized on SAT and TentaGel are following: SAT resin, peptide 1 (crude yield after synthesis 88%), (crude yield after resin cleavage 79%), (yield after HPLC purification 75%), peptide 2 (crude yield after synthesis 85%), (crude yield after resin cleavage 81%), (yield after HPLC purification 78%), peptide 3 (crude yield after synthesis 83%), (crude yield after resin cleavage 80%), (yield after HPLC purification 76%); TentaGel resin, peptide 1 (crude yield after synthesis 86%), (crude yield after resin cleavage 78%), (yield after HPLC purification 74%), peptide 2 (crude yield after synthesis 85%), (crude yield after resin cleavage 82%), (yield after HPLC purification 79%), peptide 3 (crude yield after synthesis 80%), (crude yield after resin cleavage 76%), (yield after HPLC purification 72%). The purity of endothelin receptor antagonists (peptides 1 and 2) were tested by HPLC using C-18 column. The purities of peptide 1 synthesized on SAT and TentaGel resins are shown in Supporting Information Figure 4a and b, respectively, and those of peptide 2 synthesized on SAT

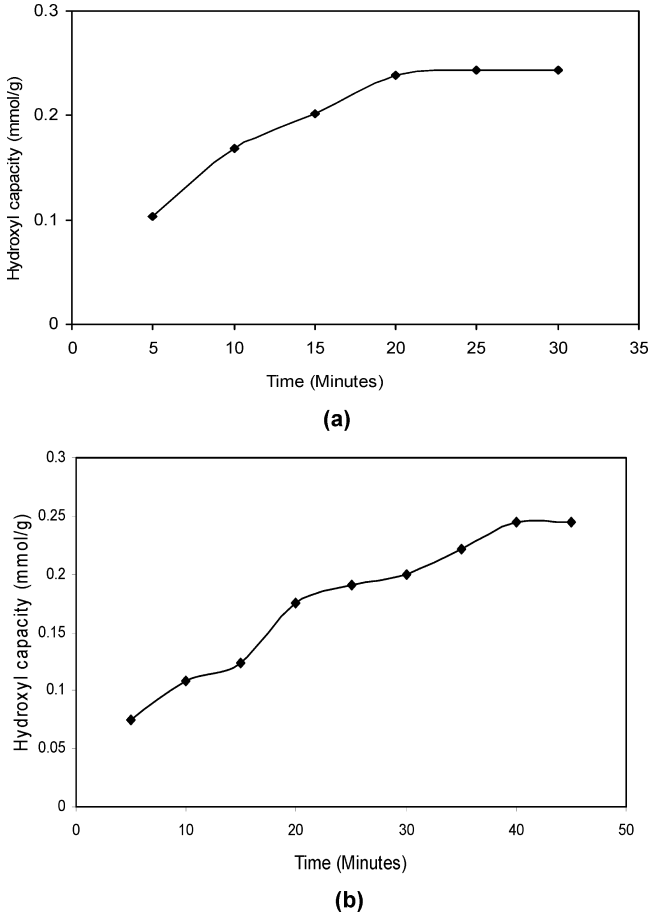


Figure 5. Time-dependent Fmoc−tryptophan deprotection of (a) SAT and (b) PS-DVB resin.

and TentaGel resins are shown in Supporting Information Figure 5a and b, respectively. Further confirmation of the peptide 1 synthesized on SAT and TentaGel resin by MALDI TOF MS is shown in Supporting Information Figure 6a and b. The peptide 2 synthesized on SAT resin was confirmed by Maldi Toff MS as shown in Supporting Information Figure 7. Peptide 3 synthesized on SAT resin and TentaGel was analyzed by HPLC (Supporting Information Figure 8a and b), and the purity of peptide synthesized on SAT was further confirmed by MALDI TOF MS (Supporting Information Figure 9).

Conclusion

The demonstrated new high-loading cross-linking SAT polymers have interesting physicochemical properties, which classify them as ideal polymer supports for multistep peptide synthesis and immobilization of reagents in catalysis. The combination of high loading capacity and good swelling property in a wide range of solvents is absent in many other existing solid-phase supports. Their suitability as supports for peptide synthesis was demonstrated by the synthesis of biologically significant endothelin family peptides. An additional support of tri(propyleneglycol) diacrylate, along with acryloyloxyhydroxypropyl methacrylate, provides high mechanical stability and easy diffusion of reagents and solvents through the resin matrix. The new SAT resin can be effectively and conveniently used for the chemical synthesis of hydrophobic peptides.

Experimental Section

FT-IR spectra were recorded on a Nicolet 5700 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. HPLC was performed on a Pharmacia Akta purifier instrument (C-18 reversed phase prep. HPLC). Mass spectra of the peptides were performed using Kratos MALDI-TOF MS instrument.

Synthesis of Styrene−Acryloyloxyhydroxypropyl Methacrylate−Tripropyleneglycol Diacrylate (SAT Resin). Styrene was washed with 1% NaOH solution and distilled water to remove inhibitors. A four-necked reaction vessel equipped with a thermostat, Teflon stirrer, water condenser and nitrogen inlet were used for the polymerization. A net volume of 1% solution of poly(vinyl alcohol) ($M_w \approx 75\,000$ 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 4 mol % SAT resin, the monomer, styrene (94 mol %), 10.77 mL, and cross-linkers acryloyloxyhydroxypropyl methacrylate (4 mol %), 0.752 mL, tri(propyleneglycol) diacrylate (2 mol %), 0.583 mL, were mixed with toluene (8 mL) and added to the reaction vessel. Different cross-linking densities of SAT resins were prepared; in all cross-linking densities, the percentage of tri(propyleneglycol) diacrylate was kept as 2 mol % and the monomer styrene and functional cross-linker acryloyloxyhydroxypropyl methacrylate were changed. For the preparation, 2 mol % SAT resin, 96% styrene, 2 mol % tri(propyleneglycol) diacrylate, and 2 mol % acryloyloxyhydroxypropyl methacrylate were taken, likely in 8 mol % resin; 90% styrene, 2 mol % tri(propyleneglycol) diacrylate, and 8 mol % acryloyloxyhydroxypropyl methacrylate were taken, respectively. For the preparation of SAT resin the 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, and the reaction vessel was sealed with a water condenser on one side and a rubber septum on the other. The system was kept under continuous flow of N_2 gas. The temperature of the reaction mixture was maintained at 80 °C using a thermo stated oil bath and the reaction were continued for 8 h. The copolymer was obtained as beads of 100−200 mesh size and washed thoroughly with hot water (to remove the stabilizer), acetone (3 × 50 mL), toluene (3 × 50 mL), and methanol (3 × 50 mL). The copolymer was further purified by Soxhlet extraction with DCM and MeOH and dried under vacuum at 40 °C (P_2O_5) for 10 h to yield 7 g of dry resin. IR (KBr, cm^{-1}): 1718 (C═O) 1122 (C−O), 3464 (O−H). ^{13}C CP-MAS NMR: 130.88, 148.83, 65.42, 43 ppm.

Hydroxyl Capacity of the Resin. The resin (100 mg) was acetylated with measured amount of acetic anhydride−piperidine mixture (1:4, 3 mL) for 6 h. Ten milliliters distilled water was added, and the reaction mixture was refluxed for 3 h. The mixture was cooled and filtered, and the acetic acid formed was back-titrated with standard (0.1 N) NaOH. A blank titration was also carried out. From the titer values,

hydroxyl capacity of the resin was calculated. The hydroxyl capacity of resin was 0.255 mmol/g.

Hydroxyl group capacity of the resin was also calculated by esterification with Fmoc-Gly (3 equiv) activated by MSNT (3 equiv) dissolved in dry DCM in presence of the base *N*-methylimidazole (2.25 equiv) and measuring the UV-absorbance of the adduct of dibenzofulvene and piperidine formed by treatment of accurately weighed polymer sample with 20% piperidine in DMF. Both the techniques gave concordant values.

Conversion of Hydroxyl Group to Chloro Group. Four mole percent SAT resin (1 g, 0.255 mmol) was suspended in DCM (25 mL). After 1 h, excess solvent was filtered off. To the swollen resin thionyl chloride (0.185 mL, 2.55 mmol) was added. The reaction was heated at 55 °C with occasional swirling for 6 h. The reaction mixture was cooled, washed with THF (5 × 15 mL), THF/H_2O (1:1, 5 × 15 mL), THF (5 × 15 mL), DCM (5 × 15 mL), methanol (5 × 15 mL), and ether (5 × 15 mL), and dried under vacuum. The above procedure was followed for chlorination of resins with other cross-linking densities; the reactions were carried out using 10-mmolar excess of the reagent with respect to hydroxyl capacity of the resin. The resin was found to have a capacity of 0.253 mmol chlorine/g. IR (KBr): 1718, 1122 cm^{-1}.

Estimation of Chloro Groups. The chlorinated resin (100 mg) was digested with pyridine (5 mL) in a Kjeldahl's flask for 5 h at 100 °C. Pyridinium chloride thus formed was quantitatively transferred to a conical flask using 50% acetic acid (30 mL). The transferred solution was acidified with conc. HNO_3 acid (5 mL), and the standard silver nitrate solution (0.1 N, 10 mL) was added to this mixture. The excess silver nitrate was determined by back-titration with standard ammonium thiocyanate solution (0.1 N) using ferric alum as an indicator until a dark brown color was obtained. A blank reaction was also performed. From the titer values, the halogen capacity of the resin was calculated as 0.253 mmol/g, by the above-mentioned Volhard's method.[20]

Amination. Four mol % chloro resin (1 g, 0.253 mmol) was swelled in excess NMP for 1 h. The resin was filtered, potassium phthalimide (0.468 g, 2.53 mmol) in NMP (20 mL) was added, and the reaction mixture was stirred at 120 °C for 12 h. The resin was collected, washed with NMP (5 × 15 mL), dioxane (5 × 15 mL), ethanol (5 × 15 mL), methanol (5 × 15 mL), and ether (5 × 15 mL), and dried in vacuum. The resin was suspended in distilled ethanol (20 mL); hydrazine hydrate (78 μL, 2.53 mmol) was added, and the mixture was refluxed for 9 h. The resin was collected by filtration, washed with hot ethanol (5 × 15 mL), methanol (5 × 15 mL), and ether (5 × 15 mL), and dried under vacuum. IR (KBr): 3420 cm^{-1} (N−H).

Estimation of Amino Groups. The resin (20 mg) was coupled with Fmoc-Gly (3.5 equiv) using HOBt (3.5 equiv), HBtU (3.5 equiv), and diisopropylethylamine (3.5 equiv) in DMF (7 mL) at room temperature, and the mixture was gently stirred for 2 h. The resin was filtered off, washed with DMF and ether, and dried under vacuum. The Fmoc amino acid attached resin (2.5 mg) was suspended in 20% piperidine in DMF (3 mL), and the mixture was stirred for 30 min. The filtrate of the reaction mixture, together with the washing filtrate, was collected .The resin loading capacity was obtained based on the UV absorption of the diluted solution at 290 nm. The amino capacity of the resin was also calculated by picric acid titration method.[21] Both methods give the concordant values of 0.252 mmol/g.

Swelling Studies. One gram of resin was accurately weighed and taken in a syringe fitted with a sintered Teflon filter. The solvent was allowed to flow through the resin for 30 min in the syringe with constant suction of flow rate 1 mL/min. The outlet of the syringe was closed and the resin was suspended in the solvent for 1 h. The swollen resin was compressed with the piston of the syringe, and the pressure was slowly released. The volume of the resin at this point was noted and related to the sample weight to obtain the resins swelling abilities. The experiment was repeated to ensure reproducible values. Different cross-linking densities (2, 4, and 6 mol %) of SAT was evaluated. The same experiment was carried out with 2 mol % SAT, PS-DVB, and TentaGel resin for their swelling comparison. Weight increase of the solvent swollen resin beads were also noted and compared with the dry resin.

Stability Studies. The chemical stabilities of the resin were carried by taking 100 mg of resin separately in each container and which was stirred separately with different reagents, such as hydroxyl amine (10 mL), aqueous ammonium hydroxide (10 mL), 20% piperidine in DMF (10 mL), 30% TFA in DCM (10 mL), and 100% TFA (10 mL). After 48 h, the resin samples were filtered, washed with ethanol, water, acetone, DCM, dioxane, and ether (each 3× 10 mL), and dried, and IR (KBr) spectra of these resins were compared with that of the original resin.

Derivatization of SAT Resin Using 4-Hydroxymethylphenoxyacetic Acid (HMPA) Linker. Amino SAT resin (1 g, 0.252 mmol/g) was swelled in DMF. After 1 h the resin was washed with 10% DIEA in DCM (5 × 50 mL), DCM (5 × 50 mL), and DMF (5 × 50 mL). HMPA (160.70 mg, 3.5 mmol), HBTU (334.5 mg, 3.5 mmol), HOBt (119 mg, 3.5 mmol), and DIEA (159 μL, 3.5 mmol) were added to preswollen amino SAT resin (1 g, 0.252 mmol) in DMF, and the reaction mixture was kept at room temperature for 1 h with occasional stirring. The resin was filtered, washed with DMF (5 × 15 mL), MeOH (5 × 15 mL), and ether (5 × 15 mL), and dried under vacuum. The resin was negative to the sensitive Kaiser test showing the 100% attachment of the anchoring group.[22]

Esterification of Fmoc-Amino Acid to Polymer Support Using MSNT. The cross-linked resin was allowed to swell in dry DCM. Dry Fmoc amino acid was dissolved in dry DCM. This solution was transferred to a stoppered flask containing MSNT. The mixture was immediately added to the swollen resin. After 30 min, the reactants were washed off with DCM, DMF, MeOH, and ether (5 × 25 mL each). The Fmoc amino acid attached HMPA resin (2.5 mg) was suspended in 20% piperidine in DMF (3 mL), and the mixture was stirred for 30 min. The filtrate of the reaction mixture together with the washing filtrate was collected. The resin loading capacity was obtained based on the UV absorption of the diluted solution at 290 nm. The resin was found to have hydroxyl capacity of 0.249 mmol/g.

Time-Dependent Fmoc Deprotection. About 250 mg of the Fmoc-amino acid anchored resin was treated with 20% piperidine/DMF (10 mL). About 10 mg of the resin was withdrawn from the reaction mixture at 5 min interval up to 40 min. The resin was washed with DMF (5 × 10 mL), MeOH (5 × 10 mL), and ether (5 × 10 mL) and dried. Accurately weighed resin was treated with 0.1 M picric acid, and the extent of Fmoc deprotection was measured from the OD of the picrate adsorbed on the resin at 358 nm. This was further confirmed by suspending 3 mg of the partially Fmoc cleaved resin in 3 mL 20% piperidine in DMF for 30 min. The percentage cleavage was estimated by measuring the OD of the dibenzofulvenepiperidine adducts at 290 nm.

General Procedure for Peptide Synthesis and Cleavage. The peptides were synthesized using Fmoc amino acids. All the Fmoc amino acids were coupled to the C-terminal amino acid attached to the resin by using HBTU and HOBt in presence of DIEA. In a typical coupling step, HBTU and HOBt were added to the Fmoc amino acid dissolved in DMF. The mixture was stirred and added to the resin swollen in DMF, and the reaction was continued for 30 min. The extent of coupling was monitored by Kaiser test. Fmoc protection was removed by using 20% piperidine in DMF. After each coupling and deprotection steps, the resin was washed with DMF (5 × 50 mL). When desired sequence of amino acids were attached to the rein, they were washed with DMF (5 × 50 mL), MeOH (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.

Acyl carrier protein synthesis was carried out by weighing equal amount of SAT and PS-DVB polymer supports in two silanized 15 mL glass reaction vessel containing a sintered filter on one side and a receiving adapter fitted with a calcium chloride guard tube on the other. The synthesis was followed by Fmoc strategy. To the preswollen polymer matrix, a 2 equiv excess of HMPA linker, along with HBTU, HOBt, and DIEA, was added for the SAT resin, and a 3 equiv excess was added for for PS-DVB. Complete incorporation of linker was monitored by Kaiser test. The C-terminal attachment was carried out by MSNT coupling strategy, under inert conditions using the following ratio of 1:1:0.75 Fmoc amino acid/MSNT/methylimidazole in dry DCM. Quantitative coupling was measured by spectrophotometric measurement of dibenzofulvene−piperidine adduct. Further coupling reactions were carried out using 2 equiv excess of Fmoc amino acid, HBTU, HOBt, and DIEA in DMF for SAT resin and 3 equiv excess for PS-DVB.

Endothelin sequence coupled peptidyl SAT resin was suspended in a mixture of cleavage cocktail having TFA (6.52 mL), thioanisol (400 μL), ethanedithiol (200 μL), phenol (400 μL), triisopropylsilane (80 μL), and double distilled water (400 μL). The mixture was kept at room temperature for 4 h. The SAT resin was filtered off, washed with fresh TFA, rinsed with DCM and vacuum evaporated to obtain a thick oily residue. The peptide was precipitated as white powder by addition of ice cold ether, and it was washed thoroughly with cold ether (5 × 10 mL) to remove the scavengers. The ACP sequence-coupled peptidyl SAT resin was suspended in cleavage cocktail containing TFA (7.52 mL), ethanedithiol (200 μL), triisopropylsilane (80 μL), and double distilled water (200 μL), and the mixture was kept at room temperature. After 4 h, the suspension was filtered and washed with TFA, and the filtrate was concentrated under reduced pressure. The peptide was precipitated by adding ice cold ether, was washed with ether (5 × 10 mL), and was dried by lyophilization.

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Supporting Information Available. Chemical stability of SAT resin and synthesized peptides' HPLC and MALDI TOF MS, regarding its purity and mass. This information is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations

AHMA	3-(acryloyloxy)-2-hydroxypropyl methacrylate
TPGDA	tri(propyleneglycol) diacrylate
PVA	poly(vinyl alcohol)
DIEA	diisopropylethylamine
HMPA	4-hydroxymethyl phenoxyacetic acid
HBTU	2-(1*H*-benzotriazol-1-yl) 1,1,3,3-tetramethyluronium-umhexafluorophosphate
HOBt	1-hydroxybenzotriazole
MSNT	1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole

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