Kinetics of Amide Formation on 1,4-Butanediol Dimethacrylate Crosslinked Polystyrene Resin: A Comparison with PS-DVB Resin

M. Roice,[1,2] V. N. Rajasekharan Pillai[1,3]

[1]*School of Chemical Sciences, Mahatma Gandhi University, Kottayam-686 560, Kerala, India*
[2]*Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500, Valby-Copenhagen, Denmark*
[3]*National Assessment and Accreditation Council (NAAC), Rajajinagar, Bangalore, India*

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ABSTRACT: An efficient crosslinked polymer support was synthesized by introducing 1,4-butanediol dimethacrylate crosslinker to a polystyrene network using aqueous suspension polymerization technique. The support was functionalized with aminomethyl groups using chloromethyl phthalimide. The kinetics of amide bond formation on 1,4-butanediol dimethacrylate crosslinked polystyrene (PS-BDODMA) polymer were carried out using the attachment of Rink amide linker as a model reaction. The efficiency of the support was tested and compared with Merrifield resin by following different steps involved in the synthesis of a 14-residue model peptide, Lys-Ile-Asn-Thr-Asn-Ala-Ser-Trp-His-Ala-Asn-Arg-Thr-Ala-NH_2, under the same synthetic conditions. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 88: 2897–2903, 2003

Key words: kinetics; peptides; synthesis; resins

INTRODUCTION

The introduction of the principle of amino acid incorporation on insoluble PS-DVB polymer support marked the milestone in organic and combinatorial synthetic chemistries, especially in the field of peptide, small protein and oligonucleotide research.[1] The macromolecular network of the support has a large influence on the outcome of various chemistries involved at various stages of the synthesis.[2] This was far more evident when larger molecules like polypeptides, small proteins and oligonucleotides were constructed on the support.[3] Extensive research on PS-DVB resins showed that the insoluble support does actually have a dynamic influence on the synthesis of peptides.[4] The hydrophobic macromolecular network of the PS-DVB support had a negative influence on various chemical reactions between the reactive centers of the polymer and the reactants, especially when the reactions were carried out in polar organic solvents.[5] The formation of truncated peptides by incomplete coupling and deprotection steps and the regrowth of partially complete sequences with the formation of deletion sequences poses a major problem in the overall yield of the pure peptide.[4] In order to overcome various drawbacks of divinyl benzene crosslinked polystyene (PS-DVB) resin, a number of new polymer supports for polypeptide synthesis have been developed and tested. These include polyamides, carbohydrates, polyethyleneglycol–polyacrylamide (PEGA) and crosslinked ethoxylate acrylate resin (CLEAR).[5–8]

Compared to other supports, styrene based polymer supports show high mechanical and chemical stability in various reagents and solvents that are used for polypeptide synthesis.[9] The various problems associated with PS-DVB resin could be overcome by prudent choice of the crosslinker that brings optimum hydrophobic-hydrophilic balance, various chemical reagents, and the solvent used in the synthesis. In order to reduce the hydrophobicity of PS-DVB resin, we developed a new series of styrene based supports by introducing various crosslinkers like tetraethyleneglycol diacrylate, 1, 6-hexanediol diacrylate and 1,4-butanediol dimethacrylate to a polystyrene network.[10–14] The degree of crosslinking of these resins had an influence on both the hydrophilicity and the swelling characteristics of the resin in various solvents. In this article we discuss the various parameters that can influence the performance of PS-BDODMA resin as a solid support for polypeptide synthesis, performance in the stepwise incorporation of amino acid and reaction kinetics involved in the amide bond formation. We have found that PS-BDODMA resin was stable under the various reaction conditions used in the peptide synthesis and also stable under the conditions used to release synthesized peptide from the support. A 14-residue model peptide was synthesized on PS-BDODMA and PS-DVB resin under similar synthetic conditions. Purity and analysis of these peptides by various techniques revealed that PS-BDODMA support can be used as a better support for

Correspondence to: M. Roice (mroice@crc.dk).

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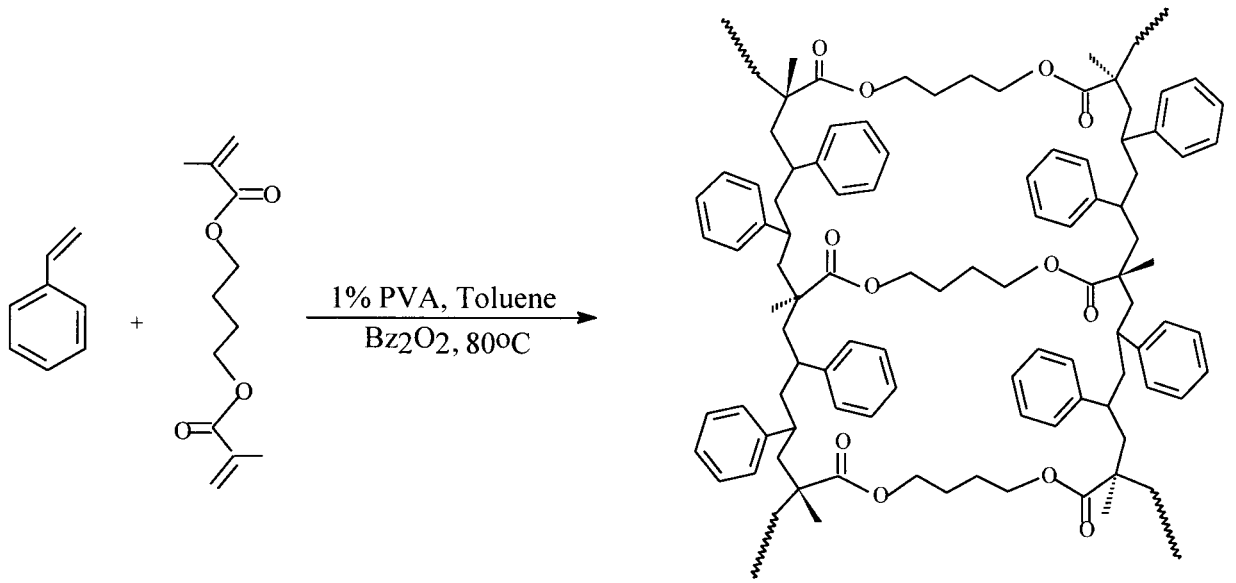


Scheme 1 Synthesis of PS-BDODMA resin.

batchwise and continuous-flow peptide synthesis compared to PS-DVB resin.

EXPERIMENTAL

Materials

Styrene, BDODMA, poly(vinyl alcohol) (PVA, MW ~ 75 000), trifluoroacetic acid (TFA), chloromethyl phthalimide and phenol were purchased from Aldrich Chemical Co. (Milwaukee, USA). Piperidine; 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU); 1-hydroxybenzotriazole (HOBt); Fmoc-amino acids and p-[(*R,S*)-α-[1-(9H-fluorene-9-yl) methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid (Rink amide handle) were purchased from Novabiochem Ltd., Nottingham, UK. Thioanisole, ethanedithiol, and diisopropyl ethylamine (DIEA) were purchased from Sigma Chemicals Co., St. Louis, USA. All solvents used were of high-performance liquid chromatography (HPLC) grade.

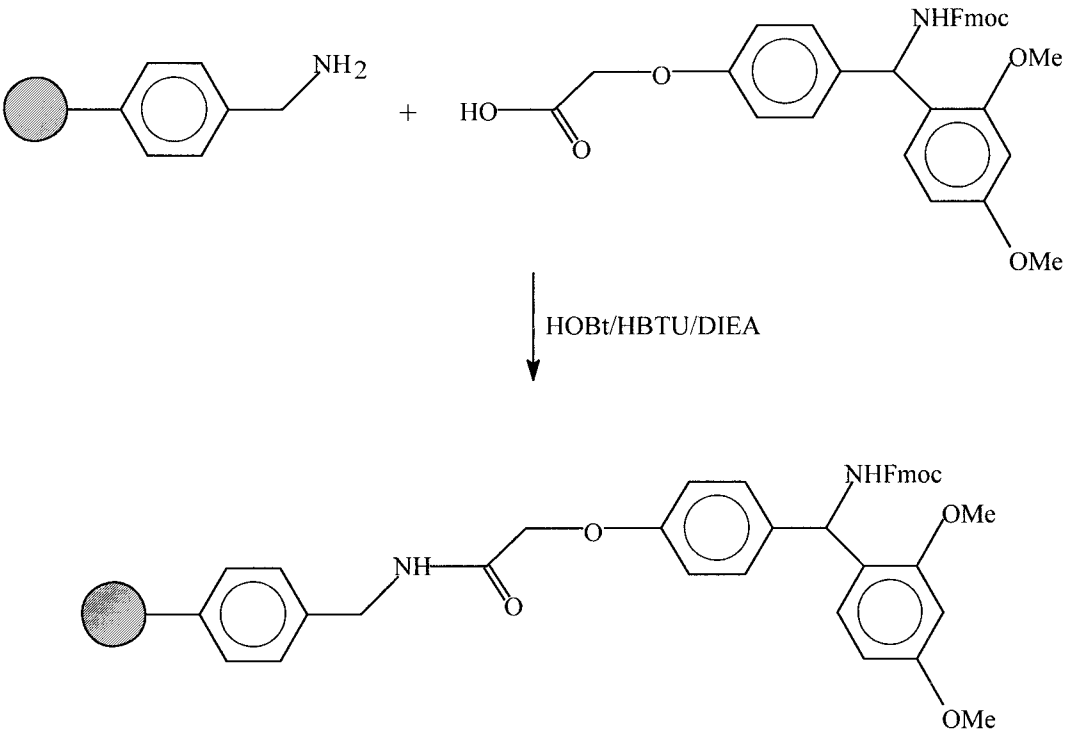
IR spectra were recorded on a Shimadzu IR 470 spectrometer using KBr pellets. The ^{13}C CP-MAS solid-state NMR measurements were conducted on a Bruker 300 MSL CP-MAS instrument operating at 75.47 MHz. HPLC was done on a Pharmacia Akta purifier instrument using C-18 reverse phase semi prep. HPLC column. The amino acid analysis was carried out on an LKB 4151 Alpha plus amino acid analyzer. Mass spectra of peptides were obtained with a Kratos PC-Kompact MALDI TOF MS instrument.

Polymer synthesis and functionalization

The 1,4-butanediol dimethacrylate crosslinked polystyrene polymer support was synthesized according to the literature procedure.[13] The PS-BDODMA resin (5 g) was swelled in dimethylformamide (DMF) (50 mL). After one hour, excess DMF was removed and the swollen polymer was placed in a three-necked 100 mL round-bottom flask equipped with nitrogen inlet, addition funnel, mechanical stirrer, and reflux condenser and heating mantle. Chloromethyl phthalimide (195.6 mg, 1 m*M*) and anhydrous $ZnCl_2$ (0.1*M* in dry THF, 1 mL) were dissolved in DMF (30 mL) and the mixture was added to the resin. The suspension was refluxed for four hours under the nitrogen atmosphere. The reaction mixture was cooled and the suspension was filtered and washed with dichloromethane (DCM) (5 × 30 mL), dioxane (5 × 30 mL), ethanol (5 × 30 mL), and methanol (5 × 30 mL). The dried resin was suspended in ethanol (20 mL), and hydrazine hydrate (100 μL, 2 m*M*) was added. The reaction mixture was refluxed for eight hours. The resin was filtered and washed with ethanol (5 × 30 mL) and methanol (5 × 30 mL). The product resin was dried in vacuum. The amino capacity of the resin was 0.19 mmol/g, as estimated by the picric acid titration method.[16] IR (KBr) revealed the following: 1720 cm^{-1}, 1520 cm^{-1}, 1480 cm^{-1}, 755 cm^{-1}, and 700 cm^{-1}. ^{13}C CP-MAS NMR results were: 145.30 ppm, 136 ppm. The aminomethyl PS-DVB resin (2 g) was synthesized by using the above procedure. The amino capacity of the resin was estimated to be 0.17 mmol/g.

Amide bond formation: rink amide linker attachment

Resins (0.2 mmol) were pre-swelled in 20 mL anhydrous DCM for 30 min at room temperature. For the Rink amide linker attachment on resins, a solution was



Scheme 2 Aminolysis of PS-BDODMA and PS-DVB resins with Rink amide linker.

prepared in a minimum amount of DCM using 4 m*M* Rink amide linker, 4 m*M* DIEA, 4 m*M* HBTU, and 4 m*M* HOBt, and the solution was added to the swollen resin. The mixture was shaken well at regular time intervals, 10–20 mg of the resin were taken out, and the reaction was stopped immediately by washing with DCM. The samples were further washed with DMF (6 × 30 mL), methanol (6 × 30 mL), DCM (6 × 30 mL) and ether (6 × 30 mL), and dried under vacuum. The reaction was allowed to continue for one hour, and the quantitative reaction was estimated by ninhydrin test. The resin was filtered and washed with DMF (6 × 30 mL), dioxane/water (1:1, 6 × 30 mL), MeOH (6 × 30 mL) and ether (6 × 30 mL). The resin was collected and dried in a vacuum.

Measurement of amide bond formation by Fmoc–analysis

The amount of attached Rink amide linker on the resin was measured by Fmoc–analysis. Resins of precise weight were treated with 3 mL of 20% piperidine/DMF solution at room temperature for 20 min. The absorbance of the solution at 290 nm was measured using a 1 cm quartz cuvette.

PS-DVB-Rink amide resin (0.15 mmol/g) was also synthesized by the same procedure described above. A second one-hour coupling was performed for the quantitative reaction.

Attachment of Fmoc-Ala to rink amide resins

Fmoc-Ala (102 mg, 0.33 m*M*), HBTU (125 mg, 0.33 m*M*), HOBt (45 mg, 0.33 m*M*) and DIEA (5 μL, 0.33 m*M*) were added to the pre-swollen rink amide resin (600 mg, 0.11 mmol) in DMF. The reaction mixture was kept at room temperature for one hour. The resin was filtered, washed thoroughly with DMF (6 × 30 mL), DCM (6 × 30 mL), methanol (6 × 30 mL) and

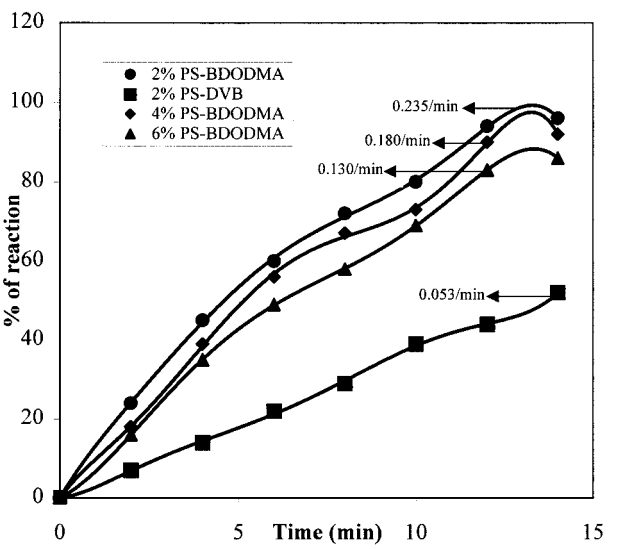


Figure 1 Time-course of Rink amide linker attachment on PS-BDODMA and PS-DVB resins in DCM.

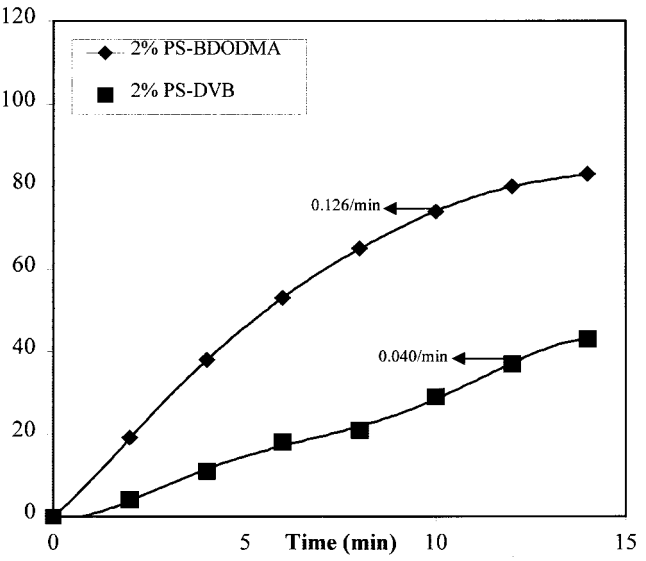


Figure 2 Time-course of Rink amide linker attachment on 2% PS-BDODMA and 2% PS-DVB resins in DMF.

ether (6 × 30 mL) and dried in a vacuum. The extent of attachment of amino acid was estimated by adding a 20% piperidine in DMF (3 mL) solution to 10 g of the resin. After 20 min OD of the solution was measured at 290 nm. From the OD value, the amino capacity of the resin was calculated. The aminolysis reaction was 96.2% for PS-BDODMA-Rink amide resin whereas the reaction was 52.2% for PS-DVB resin after 14 min. For a quantitative incorporation of the amino acid to the PS-DVB resin, two one-hour coupling reactions were required. The amino capacity of PS-BDODMA-Rink amide-Ala resin was calculated to be 0.17 mmol/g. The amino capacity of PS-DVB-Rink amide-Ala resin was calculated to be 0.14 mmol/g.

General procedure for peptide synthesis

The peptides were synthesized manually using a silanized glass reaction vessel. The reaction vessel had a sintered ware filter on one side and a receiving adapter that could be fitted with a calcium chloride guard tube on the other side. All peptides were synthesized using Fmoc-amino acids. The Fmoc-amino acids were coupled to the C-terminal amino acid attached resin (1 equiv) by the HOBt active ester method. In a typical coupling step, HOBt (3 equiv), HBTU (3 equiv) and DIEA (3 equiv) were added to the pre-swollen resin in DMF, and the coupling reaction was continued for 30 min. The extent of coupling was monitored by Kaiser test.[15] Fmoc-protection was removed by a solution of 20% piperidine in DMF. After each coupling and Fmoc-deprotection step, the resin was washed with DMF (6 × 30 mL). After the synthesis, the peptidyl resin was washed with DMF (6 × 30 mL), methanol (6 × 30 mL), and ether (6 × 30 mL) and dried under vacuum.

The peptide was cleaved from the polymer support with a mixture of TFA (2.55 mL), thioanisole (150 μL), ethanedithiol (75 μL), phenol (75 μL) and double distilled water (150 μL). The reaction mixture was kept at room temperature. PS-BDODMA resin requires two hours for peptide cleavage, PS-DVB resin requires four hours. The polymeric material was filtered and washed with neat TFA (1 ML) and DCM (10 mL). The combined filtrate and washings were vacuum-evaporated at 40°C until the filtrate became an oily residue. The peptide was precipitated as a white powder by adding ice-cold ether. The precipitate was washed with ether (6 × 30 mL) to remove scavengers. The peptide was dissolved in glacial acetic acid and reprecipitated by adding cold ether. The precipitate was again washed thoroughly with cold ether (6 × 30 mL). The purity of the crude peptide was checked by HPLC and the identity was determined by amino acid analysis and MALDI TOF MS.

RESULTS AND DISCUSSION

The 2% PS-BDODMA and 2% PS-DVB resins were synthesized by the free radical aqueous suspension polymerization using toluene as the diluent and benzoylperoxide as the initiator. The copolymerization was achieved through the dispersion of styrene, BDODMA, toluene and benzoylperoxide mixture in an aqueous solution of 1% PVA (Scheme 1). The solution was deoxygenated by continuous flow of N_2 gas until the solution became clear. Mechanical stirring of this solution at 1000 rpm resulted in the formation of uniform droplets of the dispersed organic phase in the dispersion medium. The stabilizer poly(vinyl alcohol) solution prevented the respective droplets from coagulation. The radical initiator became solubilized in the organic phase and advanced the free radical polymerization achieved at high temperature. The chain initi-

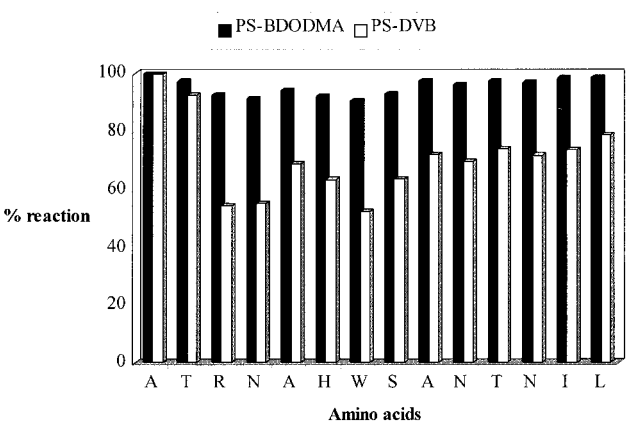


Figure 3 Extent of incorporation of amino acids in PS-BDODMA and PS-DVB resin.

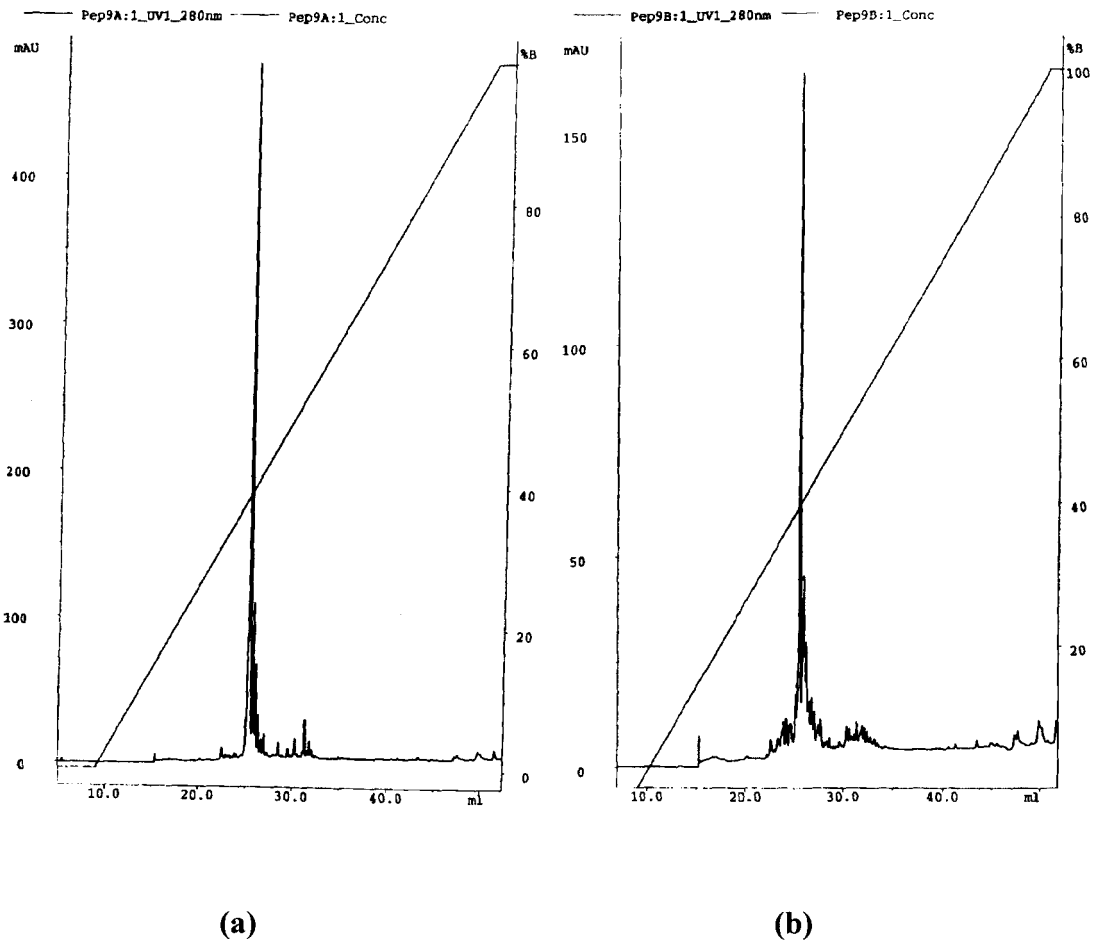


Figure 4 HPLC profile of peptide synthesized on: (a) PS-BDODMA resin and (b) PS-DVB resin using the buffer (A) 0.5 mL TFA in 100 mL water; (B) 0.5 mL TFA in 100 mL acetonitrile/water mixture (4:1). The flow rate was 0.5 mL/min and the gradients used were 0% B in 5 min and 100% B in 50 min.

ation, chain propagation and chain termination reactions proceeded in each droplet.

Many factors, such as the choice of diluent, the amount of diluent, the ratio of aqueous phase to organic phase, the size and the shape of the reaction vessel, and the stirring speed can affect the suspension polymerization and bead size of the polymer. By increasing the ratio of aqueous phase to organic phase (i.e. by reducing the volume of the diluent), a polymer of small bead size can be obtained. This can also be achieved by increasing the stirring speed. It is possible to synthesize a polymer that falls within a precise range of bead size by carefully adjusting the type of the diluent, the ratio of aqueous phase to organic phase, and the stirring speed.

The polymer synthesized was characterized by IR and ^{13}C CP-MAS NMR spectroscopic techniques. The IR spectrum of PS-BDODMA resin shows a sharp band at 1720 cm^{-1}, corresponding to the ester carbonyl of the crosslinker, in addition to the standard absorption bands of polystyrene. The solid-state ^{13}C CP-MAS NMR spectrum of PS-BDODMA resin shows an intense peak at 130.48 ppm, corresponding to the aromatic polystyrene carbons, and a small peak at 145.30 ppm, corresponding to the C-3 carbon of styrene. The peak at 42.78 ppm corresponds to the backbone methylene carbon, and the peak at 67.47 ppm corresponds to the methylene carbon of the crosslinking agent. The peak at 198.5 ppm represents the carbonyl carbon of the ester group in the polymer support.

The aminomethyl functionalized PS-BDODMA support was prepared by the derivitization of preformed resin. The aminomethyl group was introduced to the resin by treatment with chloromethyl phthalimide in the presence of $ZnCl_2$. Because of its relatively high loading of the functional group, the resin showed a decrease in swelling characteristics. This may be due to additional crosslinking that occurred during the functionalization of the resin. Amino functionality of the resin showed a positive Kaiser test.[15] The amino capacity of the resin was estimated by picric acid titration method[16] and found to be 0.17 mmol/g. IR and ^{13}C CP-MAS NMR spectroscopic methods were

used to characterize the resin. The PS-BDODMA resin showed a characteristic IR absorption band at 1720 cm^{-1}, 1480 cm^{-1} (ester) and 1520 cm^{-1} (amino). ^{13}C CP-MAS NMR shows a peak at 58.93 ppm for the methylene carbon of the aminomethyl group and a small peak in the region of 136.42 ppm for the C-6 carbon of the polystyrene ring.

In order to investigate the reactivity of the attached functional group, aminolysis of aminomethyl resins were carried out. The kinetic comparison of amide formation on various crosslinked PS-BDODMA resins and 2% PS-DVB resin were performed using the attachment of Rink amide linker as the model reaction (Scheme 2). Figure 1 illustrates the time course of independent amide formation experiments on 100–200 mesh aminomethyl PS-BDODMA resins with crosslink densities of 2%, 4%, and 6%, and on 2% PS-DVB resin using 4 mmol Rink amide linker, 4 mmol DIEA, 4 mmol HBTU and 4 mmol HOBt in a minimum amount of DCM at 25°C. The reactive intermediates formed by the Rink amide linker and HOBt/HBTU are present in approximately 20 times molar excess compared to the amino groups in the resins, and therefore the reaction can be treated as a pseudo-first order reaction.[17] The completed amide formation percentage (a_o) and the observed rate constant (k_{obs}) can be fitted with the equation

$$y = a_o(1 - e^{-k_{obs}t})$$

where y is the measured Rink amide product percentage at each time interval.

Aminolysis was followed by a measurement of the optical density of the liberated piperidine-dibenzofulvene adduct after treatment of the linker-attached resin with piperidine at two-minute intervals. It was difficult to calculate the actual rate constant because the polymer formed a separate phase in the reaction mixture. The extents of aminolysis for 2% PS-BDODMA and 2% PS-DVB resins after 14 min were 96.2% and 52.2 %, respectively. The rate constants of aminolysis for 2% PS-BDODMA and 2% PS-DVB are 0.235 min^{-1} and 0.053 min^{-1}, respectively. The comparative aminolysis of the 2% PS-BDODMA and the 4 and 6 mole % PS-BDODMA shows that the reaction rate decreases as the percentage of cross-linking increases. The calculated rate constants for 4% and 6% PS-BDODMA are 0.180 min^{-1} and 0.130 min^{-1} respectively. The high rate of reactivity is due to the flexibility and high swelling characteristics of the PS-BDODMA copolymer. The soluble reactants can easily diffuse into the polymer matrix and increase the rate of reaction. The compatibility of the polymer matrix with the reactants is also a favorable factor for the enhanced rate of reaction.

The same aminolysis reaction was performed in DMF (Fig. 2). The rate of the reaction for 2% PS-BDODMA and 2% PS-DVB were 0.126 min^{-1} and 0.040 min^{-1} respectively. The slower reaction rate and the lower conversion relative to the reaction performed in DCM could be explained by the lower swelling of the resins in DMF.

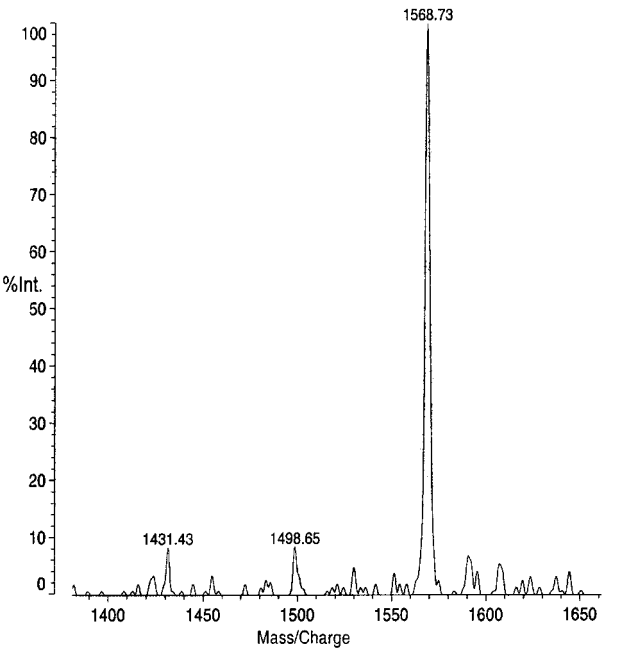


Figure 5 MALDI TOF MS of the 14-residue peptide from PS-BDODMA resin.

Solid phase peptide synthesis using PS-DVB resin is best carried out using low initial matrix loading, between 0.1 and 0.3 mmol of amino acid per gram of resin.[18] The PS-BDODMA resin with high capacity can be successfully used for the peptide synthesis, because of its high flexibility and long crosslinks.[19–21] But to insure homogeneity in comparative study, a low capacity PS-BDODMA resin was used for this synthesis. The Fmoc-Ala was introduced to the Rink amide linker attached resin by using HOBt/HBTU coupling procedure.

The PS-BDODMA-Rink amide-Ala-Fmoc resin (0.034 mmol) was used for the synthesis of a 14-residue model peptide (Lys-Ile-Asn-Thr-Asn-Ala-Ser-Trp-His-Ala-Asn-Arg-Thr-Ala-NH_2). The Fmoc protection was removed by 20% piperidine in DMF, and the subsequent amino acids were coupled successively using HOBt/HBTU/DIEA method. After the synthesis, the peptide was cleaved from the resin with TFA in the presence of scavengers. The crude peptide was obtained with 97% yield, and the purity was checked by HPLC [Fig. 4(a)]. The HPLC profile of the peptide showed only one major peak and measured the purity of the peptide at 95%. The comparative extent of incorporation of amino acids in the peptide sequence on the PS-BDODMA and PS-DVB resins are given in Figure 3. Amino acid analysis of the peptide is as

follows: Ala, 3.03 (3); Thr, 1.94 (2); Arg, 0.91 (1); Asn, 2.74 (3); His, 0.89 (1); Trp, 0.90 (1); Ser, 1.08 (1); Ile, 1.1 (1); Leu, 1.12 (1). Analysis by AMALDI TOF MS reveals a mass-to-charge ratio of 1568.73 [$(M+H)^+$, 100%], a formula of $C_{67}H_{106}N_{24}O_{20}$, and a required M^+ of 1567.731 (Figure 5).

The same 14-residue peptide was also synthesized on 2% PS-DVB-Rink amide-Ala-Fmoc resin (0.03 mmol) under the same conditions used in PS-BDODMA resin. The yield of crude peptide was 90%. The HPLC profile of the peptide is given in Figure 4(b). The purity of the peptide was 84%.

CONCLUSIONS

In conclusion, the rates of the PS-BDODMA support for chemical reactions were twice those of the Merrifield resin. The rate of amide formation was faster in DCM than in DMF. The synthetic efficiency of the resin was established by synthesizing bioactive peptides. As indicated by the experiments, the new support can be used for solid phase synthesis of peptides more effectively than Merrifield resin.

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