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Polystyrene resins cross-linked with di- or tri(ethylene glycol) dimethacrylates as supports for solid-phase peptide synthesis

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Abstract—Polystyrene resins cross-linked with di(ethylene glycol) dimethacrylate (DEGDMA) and tri(ethylene glycol) dimethacrylate (TEGDMA), DEGDMA–PS and TEGDMA–PS, were synthesized by suspension copolymerization. Four functionalized resins, chloromethyl resin, 4-hydroxymethylphenoxymethyl resin (Wang resin), 4-methylbenzhydrylamine resin (MBHA resin) and 2-chlorotrityl chloride resin, were prepared from DEGDMA–PS and TEGDMA–PS. DEGDMA–PS and TEGDMA–PS showed high reactivity in the functionalization reactions in comparison with Merrifield resin (polystyrene cross-linked with divinylbenzene, DVB–PS). DEGDMA–PS–Wang resin and TEGDMA–PS–Wang resin were used as the solid-phase support for the synthesis of a difficult sequence, the fragment of acyl carrier protein 65–74. The yields of the crude peptide synthesized using DEGDMA–PS–Wang resin, TEGDMA–PS–Wang resin and DVB–PS–Wang resin were 92.3%, 91.6% and 78.8%, respectively. The purities of the crude peptides were 85.7%, 88.1% and 73.3%, respectively.

1. Introdution

Solid-phase synthesis method has been widely used in the synthesis of peptides, proteins and small organic molecules and in combinatorial chemistry.[1–4] In the process of the solid-phase synthesis, polymer supports play a critical role.[5–7] Polystyrene cross-linked with divinylbenzene (DVD–PS) with low cross-linkage developed by Merrifield in 1960s[8] is still the most commonly used polymer support for solid-phase synthesis because of the advantages such as good mechanical and chemical stabilities and the facility of derivations with a wide variety of functional groups for substrate attachment. The use of DVB–PS resin in many cases, however, is accompanied by difficulty during synthesis. This difficulty is primarily due to the high hydrophobic character of the matrix, the short and rigid cross-linker (DVB) connecting the PS backbone and the presence of local higher cross-link density regions because of higher reactivity of divinylbenzene relative to styrene in the free radical copolymerization.[9,10] Although DVB–PS resin with low cross-linkage is highly swellable in most of the solvents used in solid-phase peptide synthesis, swelling degree in the higher cross-link density regions should be much smaller. Thus the reactivity of the functional groups in these regions must be lower due to the steric hindering effect and lower diffusion rate of the reagents, eventually leading to a low yield of the target product in the solid-phase synthesis. In order to circumvent the inherent problems associated with DVB–PS resins, new cross-linkers have been designed both to increase the flexibility of the polymer backbone to allow for better diffusion through the matrix and also to impart a variety of solvent-like properties to the resins,[7] such as tetraethyleneglycol diacrylate,[11] 1,6-hexanediol diacrylate,[12,13] 1,4-butanediol dimethacrylate,[14,15] α,ω-bis(4-vinylbenzyl) ethers of mono-, di-, tetra-, or hexa(ethylene glycol),[16] α,ω-bis(4-vinylbenzyl) ethers of 1,4-butanediol or polytetrahydrofuran,[17] tri(propylene glycol) glycerolate diacrylate[18] and glycerol dimethacrylate[19] were copolymerized with styrene to prepare polystyrene resins as supports for solid-phase synthesis. Some of the resins cross-linked by these cross-linkers have been used for solid-phase peptide synthesis and exhibited high synthetic efficiency.[11–15,18–20] An alternative to improve the hydrophobic character of DVB–PS resin is grafting poly(ethylene glycol) (PEG) onto the resin, forming the so-called TentaGels.[21] The functional capacity of TentaGel resins is, however, fairly low. In this paper, two cheap and commercially available cross-linkers, di(ethylene glycol) dimethacrylate (DEGDMA) and tri(ethylene glycol) dimethacrylate (TEGDMA), were used to prepare novel polystyrene resins,[22] DEGDMA–PS and TEGDMA–PS, as shown in Figure 1. Dimethacrylates rather than diacrylates were chosen

Keywords: Solid-phase peptide synthesis; Di(ethylene glycol) dimethacrylate; Tri(ethylene glycol) dimethacrylate; Solid support.
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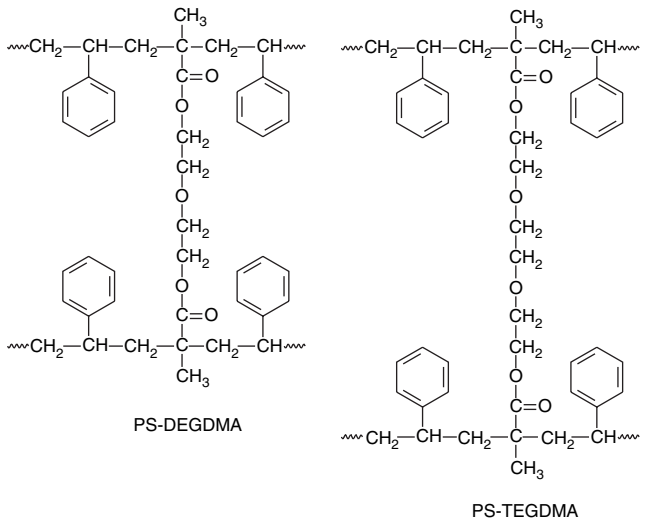


Figure 1. Structures of DEGDMA–PS resin and TEGDMA–PS resin.

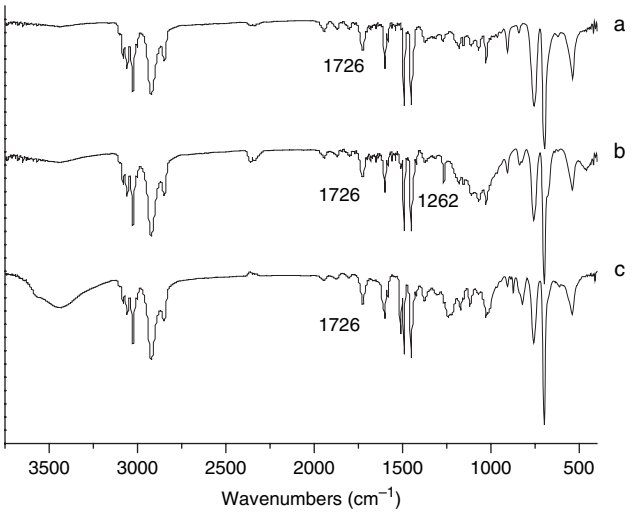


Figure 2. FTIR spectra of: (a) 3-wt % DEGDMA–PS resin, (b) clorometh-ylated 3-wt % DEGDMA–PS resin and (c) 3-wt % DEGDMA–PS–Wang resin.

for two reasons. The first reason is that polymethacrylates are much more chemically stable than polyacrylates. For example, saponification of cross-linked poly(methyl methacrylate) was much more difficult than that of cross-linked poly(methyl acrylate).[23] The second reason is that, in comparison with acrylates, methacrylates have more favourable copolymerization parameter with styrene[24] and thus the cross-linkage is more uniformly distributed in the resin made with the dimethacrylates as the cross-linker than diacrylates.

2. Results and discussion

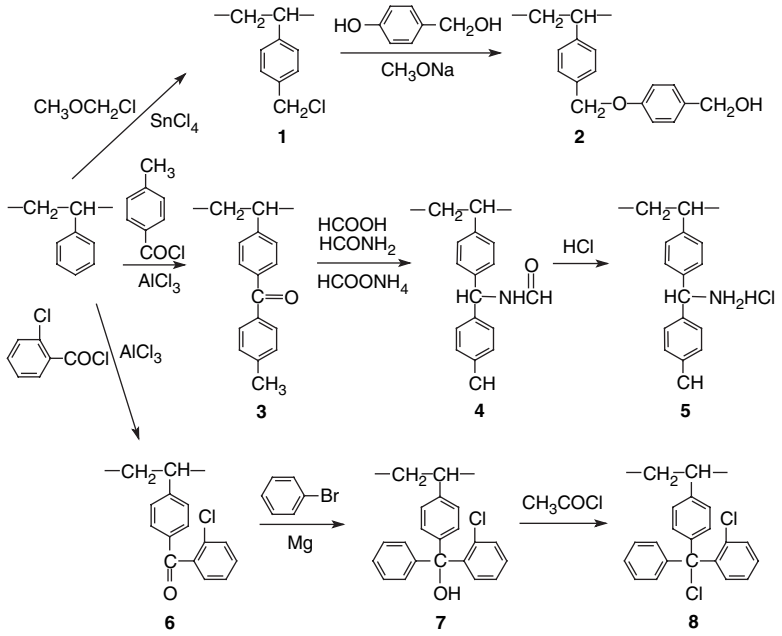
Beaded DEGDMA–PS and TEGDMA–PS resins were prepared by suspension radical copolymerization of styrene with DEGDMA and TEGDMA, respectively, with various cross-link degrees (1.6–5.3-wt % cross-linker). FTIR spectrum of 3-wt % DEGDMA–PS showed absorption at 1726 cm^{-1} (Fig. 2a), corresponding to ester carbonyl groups of the cross-liner. The swelling properties of DEGDMA–PS and TEGDMA–PS as well as 1-wt % DVB–PS gel in five solvents are listed in Table 1. It was shown that DEGDMA–PS and TEGDMA–PS resins swelled to a much greater extent than DVD–PS with a comparable amount of DVB. It was also showed that DEGDMA–PS and TEGDMA–PS resins had better swelling performance than DVB–PS resin in polar solvent compared with non-polar solvent, as indicated by ratio of swelling degree in DMF to that in CH_2Cl_2. Interestingly, DEGDMA–PS swelled to a greater extent than TEGDMA–PS with the same mole-% cross-linkage. Swelling of a gel-like resin was considered a prerequisite for facilitating reactions to occur within the solid support.[25–28] However, resins with over low cross-linkage have insufficient mechanical stability and swell excessively; excessive swelling increases the amount of solvent required for washing and the volumes of reagents needed to promote efficient synthesis. For the comparison with 1-wt % DVB–PS resin, a most commonly used support for solid-phase synthesis, 3-wt % DEGDMA–PS and 2.7-wt % TEGDMA–PS resins, which have similar or close to the swelling properties with 1-wt % DVB–PS, were used in all subsequent studies. Four functionalized resins with linkers commonly used in solid-phase peptide synthesis, chloromethyl resin (**1**), 4-hydroxymethylphenoxymethyl resin (Wang resin) (**2**), 4-methylbenzhydrylamine resin (MBHA resin) (**5**) and 2-chlorotrityl chloride resin (**8**), were prepared from 3-wt % DEGDMA–PS and 2.7-wt % TEGDMA–PS resins, as shown in Scheme 1.

The 3-wt % DEGDMA–PS and 2.7-wt % TEGDMA–PS resins were chloromethylated by Fridel–Craft alkylation

Table 1. Swelling degree (ml/g) of DEGDMA–PS and TEGDMA–PS compared to DVB–PS

Cross-linker	Cross-link degree		Dioxane	THF	DMF	Benzene	CH_2Cl_2	DMF/CH_2Cl_2[a]
	wt %	mol %						
DEGDMA	2.1	0.9	9.0	9.8	6.0	10.0	9.6	0.63
	2.4	1.0	8.3	8.6	5.4	8.8	8.4	0.64
	3.0	1.3	6.2	6.8	4.5	7.7	7.1	0.63
	4.1	1.8	5.5	5.8	4.2	6.4	6.4	0.66
	4.5	2.0	5.1	5.7	4.0	5.2	6.0	0.67
TEGDMA	1.6	0.6	7.7	9.0	5.9	9.4	9.3	0.63
	2.2	0.8	7.3	8.1	5.2	8.4	8.3	0.63
	2.7	1.0	7.0	7.4	5.0	7.7	7.4	0.68
	5.3	2.0	4.6	5.2	3.7	6.2	5.2	0.71
DVB	1.0	0.8	6.6	7.3	4.4	7.4	7.2	0.61

[a] Ratio of swelling degree in DMF to that in CH_2Cl_2.



Scheme 1. Synthetic scheme.

reaction using chloromethyl methyl ether catalyzed by anhydrous $SnCl_4$. FTIR spectrum of the resin showed absorption of chloromethyl group at 1262 cm^{-1} (see Fig. 2b) and the absorption at 1726 cm^{-1} of ester carbonyl groups of the cross-linkers remained unchanged (see Fig. 2a and b). Under the same chloromethylation conditions as shown in Section 3, the chloromethylation loadings of 3-wt % DEGDMA–PS, 2.7-wt % TEGDMA–PS and 1-wt % DVB–PS were 1.31, 1.27 and 0.85 mmol/g, respectively, indicating the higher reactivity of the resins with the two new cross-linkers as compared to DVB.

The 3-wt % DEGDMA–PS–Wang and 2.7-wt % TEGDMA–PS–Wang, were obtained by treating chloromethylated 3-wt % DEGDMA–PS (1.31 mmol Cl/g) and chloromethylated 2.7-wt % TEGDMA–PS (1.27 mmol Cl/g), respectively, with 4-hydroxybenzyl alcohol in the presence of CH_3ONa. The complete conversion was judged by no detectable residual chlorine content and disappearance of the FTIR absorption of chloromethyl group at 1262 cm^{-1} (as shown in Fig. 2b and c for DEGDMA–PS resin) of the resulting resins. The absorption at 1726 cm^{-1} of ester carbonyl groups of the cross-linkers remained unchanged, indicating that the cross-linkers were stable under the reaction condition. The loading capacities of 3-wt % DEGDMA–PS–Wang and 2.7-wt % TEGDMA–PS–Wang resins were determined by coupling Fmoc-amino acid to the resin and were found to be 0.86 and 0.80 mmol/g, respectively.

4-Methylbenzhydrylamine resin derived from 3-wt % DEGDMA–PS was prepared. The reactions were traced by FTIR spectra as shown in Figure 3. Adsorption peak of the ketone resin (**3**) at 1656 cm^{-1} (Fig. 3b) was caused by the ketone carbonyl group. When ketone resin (**3**) was transformed to formamide resin (**4**), adsorption peak at 1656 cm^{-1} disappeared and a new peak at 1695 cm^{-1} (amide carbonyl group) appeared. Complete hydrolysis of the formamide group of formamide resin (**4**) was confirmed by the disappearance of the absorption band of amide carbonyl group at 1695 cm^{-1} and 3-wt % DEGDMA–PS–MBHA (**4**) was obtained. In all the reactions, FTIR adsorption band of ester groups of the cross-linker remained unchanged, indicating that the reactions had no effect on the cross-linker introduced in the resin.

The 3-wt % DEGDMA–PS–2-chlorotrityl chloride resin (**8**) was prepared based on Scheme 1. The functionalization was confirmed by FTIR spectra, as shown in Figure 4. As in the case of the preparation of 3-wt % DEGDMA–PS–2-MBHA resin, cross-linker remained unchanged in the reactions. Under the same reaction conditions as shown in Section 3, the loadings of 2-chlorotrityl chloride resins prepared with 3-wt % DEGDMA–PS and 1-wt % DVB–PS resins were

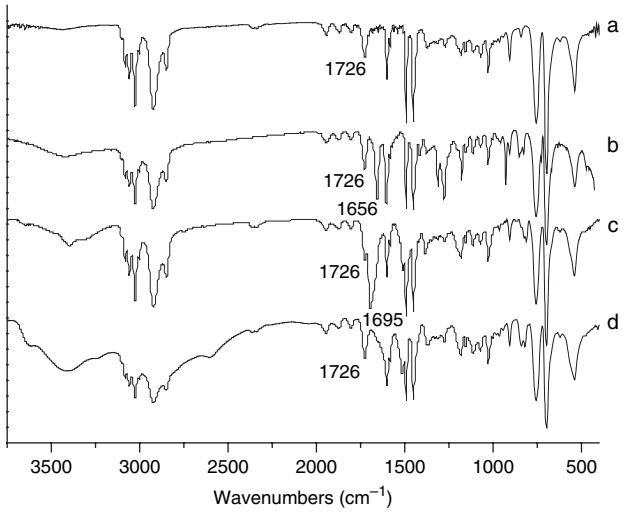


Figure 3. FTIR spectra of: (a) 3-wt % DEGDMA–PS resin, (b) 3-wt % DEGDMA–ketone resin, (c) 3-wt % DEGDMA–PS–formamide resin and (d) 3-wt % DEGDMA–PS–MBHA resin.

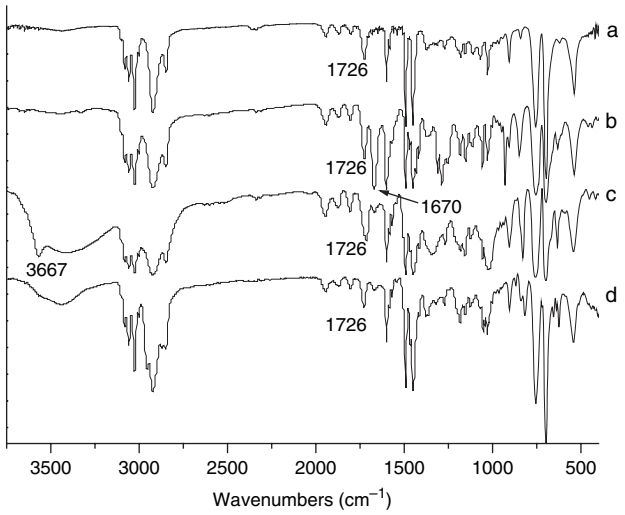


Figure 4. FTIR spectra of: (a) 3-wt % DEGDMA–PS resin, (b) 3-wt % DEGDMA–ketone resin, (c) 3-wt % DEGDMA–PS–alcohol resin and (d) 3-wt % DEGDMA–PS–2-chlorotrityl chloride resin.

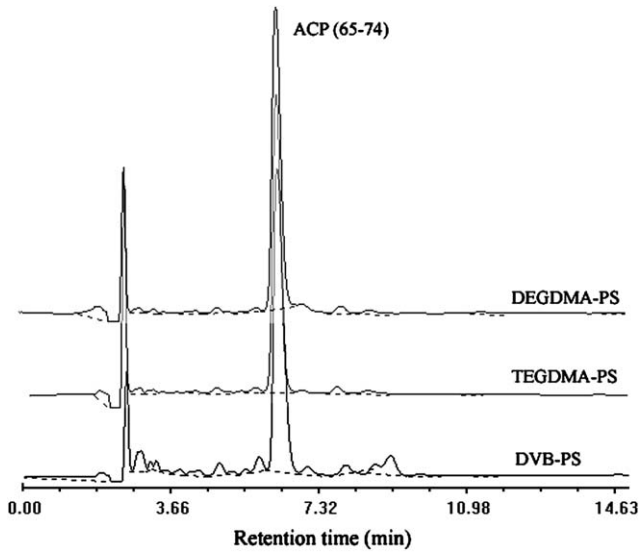


Figure 5. HPLC analysis of unpurified cleavage products. Conditions: ZORBAX SB-C18 (5 μm, 4.6×150 mm, Agilent), detected at 220 nm using 484 Tunable Absorbance Detector (Waters), 18% acetonitrile/water containing 0.05% TFA at 0.8 ml/min using 510 HPLC Pump (Waters).

determined by Fmoc-amino acid loading analysis to be 1.86 and 1.58 mmol/g, respectively.

To evaluate the application of the supports for solid-phase peptide synthesis, 3-wt % DEGDMA–PS–Wang (loading: 0.86 mmol/g) and 2.7-wt % TEGDMA–PS–Wang (loading: 0.80 mmol/g) resins were used for the synthesis of a classic difficult sequence, acyl carrier protein (65–74)[29] (ACP (65–74)) ($V^{65}Q^{66}A^{67}A^{68}I^{69}D^{70}L^{71}I^{72}N^{73}G^{74}$) by Fmoc-strategy. For comparison, 1-wt % DVB–PS–Wang resin (loading: 0.72 mmol/g) was used to synthesize the same peptide fragment in the same synthetic conditions. The yields of the crude peptide synthesized using DEGDMA–PS–Wang resin, TEGDMA–PS–Wang resin and DVB–PS–Wang resin were 92.3%, 91.6% and 78.8%, respectively, calculated on the basis of the first amino acid substitution. The purity of the acyl carrier protein fragment from each resin was tested by the HPLC on a C18 column, as shown in Figure 5. The purities of the crude peptide obtained from 1-wt % DVB–PS–Wang resin, 3-wt % DEGDMA–PS–Wang resin and 2.7-wt % TEGDMA–PS–Wang resin were 73.3%, 85.7% and 88.1%, respectively. The main peak of the HPLC chromatograms was confirmed to be ACP (65–74) by LC–MS, as shown in Figure 6. It was indicated that the purities of the peptide synthesized using 3-wt % DEGDMA–PS–Wang resin and 2.7-wt % TEGDMA–PS–Wang resin were higher than that by using 1-wt % DVB–PS–Wang resin. The 3-wt % DEGDMA–PS resin and 2.7-wt % TEGDMA–PS resin also showed high reactivity in the preparation of chloromethyl and 2-chlorotrityl chloride resins. These high reaction efficiencies may be caused by long, flexible, hydrophilic and uniformly distributed cross-linkers introduced in the resins. The 2.7-wt % TEGDMA–PS resin was slightly better than 3-wt % DEGDMA–PS resin based on the synthesis of ACP (65–74).

In summary, polystyrene resins cross-linked with DEGDMA or TEGDMA retained the advantages of DVB–PS resin, i.e., low cost, good mechanical and chemical stability and the facility of derivations with a wide variety of functional groups for substrate attachment. DEGDMA–PS and TEGDMA–PS also possessed higher reactivity in functionalization reactions. The purity of the peptide synthesized using the new supports was high when compared with the conventional DVB–PS resin.

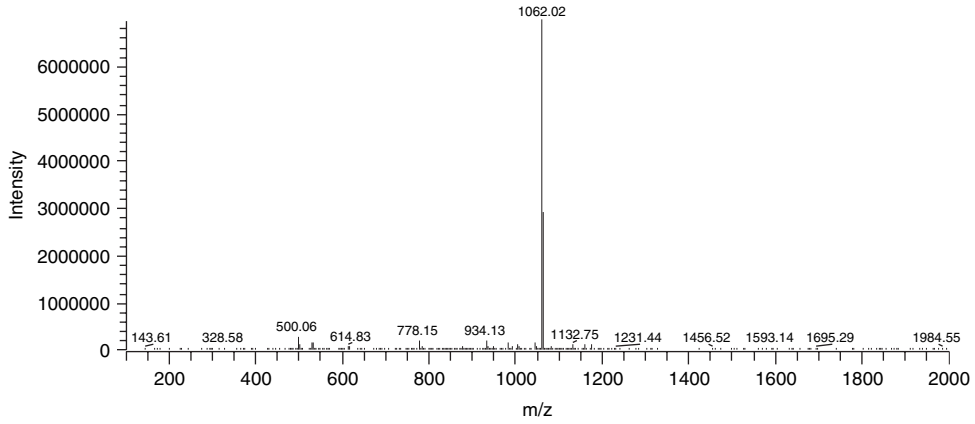


Figure 6. ESI–MS analysis of the unpurified peptide.

3. Experimental

3.1. Materials

Di(ethylene glycol) dimethacrylate (DEGDMA), tri(ethylene glycol) dimethacrylate (TEGDMA) and *N*,*N*-diisopropyl ethylamine were purchased from Aldrich. Styrene, divinylbenzene (55%), chloromethyl methyl ether, 1-wt % DVB–PS and 1-wt % DVB–PS–Wang resin (loading: 0.72 mmol/g) were from Hecheng Science & Technology Co. Ltd. 4-Hydroxybenzyl alcohol was from Lancaster. Sodium methoxide was from Fluka. Anhydrous tin tetrachloride, anhydrous aluminium chloride and magnesium were from Tianjin No. 2 Chemical Plant. 4-Toluoyl chloride, 2-chlorobenzoyl chloride, acetyl chloride and bromobenzene were from Tainyu Chemical Co. Formic acid (85%), formamide and ammonium formate were from Tianjin No. 1 Chemical Plant. Fmoc-amino acids were from Advanced ChemTech. Trifluoroacetic acid (TFA), piperidine, thioanisole, *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-dimethylamino pyridine and 1-hydroxybenzotiazole (HOBt) were from GL Biochem Ltd.

3.2. Synthesis of DEGDMA–PS and TEGDMA–PS resins

Cross-linked polystyrene resins were synthesized by suspension copolymerization. The following is a description of the synthesis of 3-wt % DEGDMA–PS as an example. A mixture of styrene (97 g), DEGDMA (3 g) and dibenzoyl peroxide (0.5 g) was suspended in 500 ml water containing 0.5% poly(vinyl alcohol) (average polymerization degree 1700, hydrolization degree 88%) with N_2 protection in a 1000 ml three-necked flask. The size of the monomer solution droplets in the aqueous phase was controlled by adjusting the stirring speed. The suspension was heated to 80 °C to initiate radical polymerization. The polymerization was carried out for 8 h at the same temperature and each droplet was converted into an individual resin bead. The polymer beads were collected by filtration and washed thoroughly with hot water to remove poly(vinyl alcohol), acetone and methanol. The resin was then extracted using 1,2-dichloroethane in a Soxhlet extractor to remove linear polymers. Beads of 100–200 mesh sizes were collected. The yield was 72%.

3.3. Swelling determination

Swelling degree was determined by the syringe method.[19] Briefly, a resin sample (500 mg) was taken in a 10-ml syringe fitted 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 and the volume of the swollen resin was recorded.

3.4. Chloromethylation of polystyrene resin

Polystyrene resin (10 g) was suspended in dichloromethane (100 ml). The mixture was stirred at room temperature for 60 min and then cooled to 0 °C. To this mixture was added a solution of chloromethyl methyl ether (10 ml) and anhydrous $SnCl_4$ (1.2 ml) in dichloromethane (50 ml). The mixture was stirred at 4 °C for 50 min. The resulting resin was washed thoroughly with dichloromethane, methanol, water and methanol and then dried under vacuum to give chloromethylated polystyrene resin (**1**).

3.5. Synthesis of 4-hydroxymethylphenoxylmethyl (Wang) resin (2)

Chloromethylated polystyrene resin (5 g) was suspended in *N*,*N*-dimethylacetamide (25 ml) and the mixture was stirred for 60 min. To this mixture was added a solution of 4-hydroxybenzyl alcohol (2.1 equiv) in *N*,*N*-dimethylacetamide (15 ml) and CH_3ONa (1.4 equiv) powder. Under protection of N_2 the mixture was stirred at 50 °C for 8 h. The resulting resin was washed thoroughly with water, 1,4-dioxane and methanol and then dried under vacuum.

3.6. Synthesis of 4-methylbenzhydrylamine (MBHA) resin (5)

The 3-wt % DEGDMA–PS resin (10 g) was suspended in dichloromethane (140 ml). The mixture was stirred at room temperature for 60 min and was then cooled to −10 °C. To this mixture was added dropwise a solution of 4-toluoyl chloride (3.1 g) and anhydrous $AlCl_3$ (2.7 g) in dichloromethane (60 ml) and the temperature of the reaction mixture was maintained below 0 °C during the dropping. The reaction mixture was heated to 20 °C and stirred for 14 h. After washing with dichloromethane, methanol, water and methanol, ketone resin (**3**) was obtained. The ketone resin (**3**) (10 g) was treated with $HCOONH_4$ (24 g), HCOOH (16 ml) and $HCONH_2$ (25 ml) in nitrobenzene (100 ml) at 160–168 °C for 40 h to form formamide resin (**4**). The resulting resin (**4**) (10 g) was then treated with concentrated HCl (20 ml) in ethanol (10 ml) at refluxing for 6 h to form 3-wt % DEGDMA–PS–MBHA resin (**5**). The loading of 3-wt % DEGDMA–PS–MBHA resin (**5**) was 0.85 mmol/g determined by Fmoc-amino acid loading method.

3.7. Synthesis of 2-chlorotrityl chloride resin (8)

Polystyrene resin (10 g) was suspended in dichloromethane (100 ml). The mixture was stirred at room temperature for 60 min and then was cooled to −10 °C. To this mixture was added dropwise a solution of 2-chlorobenzoyl chloride (12 g) and anhydrous $AlCl_3$ (9 g) in dichloromethane (50 ml) and the temperature of the reaction mixture was maintained below 0 °C during the dropping. Then the reaction was carried out at refluxing for 4 h. After washing with dichloromethane, methanol, water and methanol, ketone resin (**6**) was obtained. The resulting ketone resin (**6**) (10 g) was treated with Mg (4.5 g) and bromobenzene (25 ml) in THF (160 ml) at 65–68 °C for 48 h. Then the reaction mixture was cooled to room temperature. To this mixture was added a solution of dioxane/water/concentrated HCl (5/1/1, v/v/v, 300 ml). The mixture was stirred at room temperature for 2 h. The resulting resin was washed with dioxane/water/concentrated HCl (5/1/1, v/v/v), acetone, water, methanol and dried under vacuum to give alcohol resin (**7**). The alcohol resin (**7**) (10 g) was suspended in toluene (120 ml). To this mixture was added acetyl chloride (20 ml) and then the mixture was heated to 80 °C and stirred for 4 h. The resulting resin was washed with toluene, dichloromethane and petroleum ether and then dried under vacuum to give 2-chlorotrityl chloride resin (**8**).

3.8. Determination of amino acid loading capacity of resin by Fmoc-amino acid loading analysis

For Wang resin and MBHA resins: Resin sample (1 g) was coupled with Fmoc-Gly (5 equiv) using diisopropylcarbodiimide (5 equiv), 4-dimethylamino pyridine (50 mg) and HOBt (50 mg) in DMF (10 ml) at room temperature, and the mixture was gently stirred overnight. The resin was filtered, washed with DMF and methanol and dried under vacuum. The Fmoc-amino acid attached resin (50 mg) was suspended in 20% piperidine in DMF (5 ml) and the mixture was stirred for 30 min. The filtrate of the reaction mixture together with the washing filtrate was collected and diluted to a volume of 100 ml. The resin loading capacity was obtained based on the UV absorption of the diluted solution at 290 nm. The calibration graph was made by the UV absorption of the deprotection solution of Fmoc-Gly using the similar procedure.

For 2-chlorotrityl chloride resin: Resin sample (1 g) was treated with Fmoc-Gly (3 mmol, 0.89 g) and *N*,*N*-diisopropyl ethylamine (9 mmol, 1.95 g) in dichloromethane (10 ml) at room temperature for 2 h. The resin was filtered, washed with dichloromethane, DMF and methanol and dried under vacuum. The following operation was the same as above.

3.9. Synthesis of fragment of acyl carrier protein 65–74

Incorporation of C-terminal Fmoc-Gly to the Wang resins: Wang resin (1 g) was treated with Fmoc-Gly (3 equiv), HOBt (3 equiv) and DCC (3 equiv) in DMF (15 ml) for 8 h. The resulting resin was washed thoroughly with DMF, ethanol and DMF and was then treated with acetic anhydride (1 ml) and pyridine (1 ml) in DMF (15 ml) for 4 h and then washed as above. The loading capacity of Fmoc-Gly on the resin was estimated by measuring the absorbance at 290 nm of an accurately weighed resin in 3 ml of 20% piperidine in DMF for 30 min.

General procedure for peptide synthesis: Fmoc-Gly attached resin (0.5 g) was swelled in DMF for 30 min. To this mixture was added 20% piperidine in DMF (7 ml) and the mixture was stirred for 30 min to remove Fmoc group. The resulting resin was washed thoroughly with DMF, ethanol and DMF. Then the resin was treated next with Fmoc-amino acid (3 equiv), HOBt (3 equiv) and DCC (3 equiv) in DMF (7 ml) for 2 h. The unreacted amino groups in the resin were masked by shaking acetic anhydride (0.5 ml) and pyridine (0.5 ml) in DMF (7 ml) for 30 min. This cycle of operation was repeated for the stepwise incorporation of remaining amino acids.

Cleavage of the peptide from the resin: The peptide attached resin was treated with TFA/thioanisole/water (95/2.5/2.5, v/v/v, 10 ml) at room temperature for 4 h. The resin was filtered off and washed with TFA (two times). The combined filtrate was concentrated under vacuum and the peptide was precipitated by ether.

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