Journal of Peptide Science
J. Peptide Sci. **4**: 195–210 (1998)

PEGA Supports for Combinatorial Peptide Synthesis and Solid-phase Enzymatic Library Assays

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Received 08 July 1997
Accepted 11 August 1997

Abstract: Permeable resins cross-linked with long PEG chains were synthesized for use in solid-phase enzyme library assays. High molecular weight bis-amino-polyethylene glycol (PEG) 4000, 6000, 8000 were synthesized by a three-step reaction starting from PEG-bis-OH. Macromonomers were synthesized by partial or di-acryloylation of bis-amino-PEG derivatives. Bis/mono-acrylamido–PEG were copolymerized along with acrylamide by inverse suspension copolymerization to yield a less cross-linked resin (Type **I,** compounds **6–9**). Furthermore, acryloyl–sarcosin ethyl ester was co-polymerized along with bis-acrylamido PEG to obtain more crosslinked capacity resin (Type **II,** compounds **13–19**). *N*,*N*-Dimethylacrylamide was used as a co-monomer in some cases. The polymer was usually obtained in a well-defined beaded form and was easy to handle under both wet and dry conditions. The supports showed good mechanical properties and were characterized by studying the swelling properties, size distribution of beads, and by estimating the amino group capacity. Depending on the PEG chain length, the monomer composition and the degree of cross-linking the PEGA supports showed a high degree of swelling in a broad range of solvents, including water, dichloromethane, DMF, acetonitril, THF and toluene; no swelling was observed in diethyl ether. The PEGA resins (Type **I**) with an amino acid group capacity between 0.07 and 1.0 mmol/g could be obtained by variation of the monomer composition in the polymerization mixture. Fluorescent quenched peptide libraries were synthesized on the new polymer using a multiple column library synthesizer and incubated with the matrix metalloproteinase MMP-9 after it had been activated by 4-aminophenyl mercuric acetate resulting in 67/83 kDa active enzyme. The bright beads were separated manually under a fluorescence microscope and sequenced to obtain peptide substrates for MMP-9. After treatment with ethylene diamine, high-loaded resins (Type **II**) have been employed in continuous flow peptide synthesis to yield peptides in excellent yield and purity. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: PEGA; solid-phase enzyme assay; PEG; MMP-9; fluorescence quenched peptides libraries

INTRODUCTION

Merrifield originally used cholormethyl divinyl benzene cross-linked polystyrene resin for solid-phase peptide synthesis (SPPS) [1]. Even though this resin is widely used in SPPS there are some inherent problems in obtaining pure peptides using polystyrene supports [2,3]. Polar acrylamide supports were introduced as an alternative to polystyrene resin by Sheppard and co-workers [4]. Since then a number of polymer supports have been developed mainly for peptide synthesis [5–12]. Polyethylene glycol was grafted onto choloromethyl

Abbreviations: Abz, 2-aminobenzoic acid; ACH, α-cyano-4-hydroxy cinnamic acid; APMA, 4-aminophenyl mercuric acetate; DhbtOH, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzatriazine; DIPEA, diisopropyl ethyl amine; HMBA, 4-hydroxy benzoic acid; Hyp, hydroxyproline; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectroscopy; MCPS, multiple column peptide synthesis; MeIm, 1-methylimidazole; MSNT, 1-mesityl-sulphonyl-3-nitro-1,2,4-trizole; NEM, 4-ethyl morpholine; Pfp, 2,3,4,5,6-pentafluorophenyl; PEG, polyethyleneglycol; PEGA, polyethylene glycol–polyacrylamide copolymer; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulphonyl; POE, polyoxy ethylene; PS–PEG, polystyrene grafted with polyethylene glycol; TEMED, *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine.

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CCC 1075–2617/98/030195-16$17.50

polystyrene resin in order to increase the hydrophilicity [13–15]. In a different approach PEG was grafted to mono-dispersed chloromethyl polystyrene by anionic polymerization of the epoxide monomer [2,16]. PS–PEG resin prepared by grafting amino–PEG to modified polystyrene has also been reported [3,17]. With the event of synthetic combinatorial libraries there has been a rapid progress in solid-phase organic chemistry [18,19]. Initially, the Multi Pin method, where hundreds of peptides of known sequences are synthesized at the same time was introduced using polyethylene rods with grafted polyamide gel to react in an arrangement equivalent to the 96-well ELISA-format [20]. In the Tea Bag method, a multitude of peptide sequences were synthesized and tested for bio-activity in solution after cleavage [21]. However, these techniques are not considered to be true libraries. The split synthesis method for generating libraries was first reported for the synthesis of equimolar peptide mixtures [22,23]. This synthetic method was later used to generate iterative libraries [24] and one bead–one peptide libraries [25]. In the iterative approach, free soluble peptide mixtures are generated and the assays are carried out in solution [24]. The process is iteratively repeated to identify a single best lead compound. The one bead–one compound approach is a process where a very large number of discrete structures are screened concurrently, often resulting in the identification of one or more lead structures. There are many techniques for screening bead-bound libraries. The polymer support plays a major role in the selection of appropriate screening procedures. It has been demonstrated that conventional peptide synthesis resins do not permit the access of enzymes and other macromolecules into the interior of the polymeric support [26]. A complete subsite mapping of an endoprotease by fluorescent quenched resin-bound peptide libraries has been reported [27]. Furthermore, this method was modified for the identification of enzyme inhibitors of subtilisin Carlsberg [28] and cruzipian [29] using a D-amino acid containing peptide library. In search of a support for peptide synthesis and subsequent bio-assay using resin-bound peptide, the hydrophilic and flexible PEGA resins were developed as a superior polymer [30,31]. PEGA was originally introduced as a versatile cross-linked support for peptide and glycopeptide synthesis [32]. The cross-linked PEGA polymer matrix consists of bis-acryloyl amino terminated PEG chains polymerized to form a poly-acrylamide backbone. PEG is the major constituent of the polymer support and hence it is highly flexible and bio-compatible [33]. The polymer matrix was designed and synthesized in such a way that macromolecules such as enzymes can freely enter into the polymer network thereby facilitating solid-phase enzymatic reaction. In a comparative study, it was shown that PEGA resin is superior to many of the existing polymer supports for peptide synthesis [34]. The synthesis and physicochemical properties of different types of PEGA polymer is described in the present paper with emphasis on peptide synthesis and in particular solid-phase enzyme assays using the comparatively large matrix metalloproteinase MMP-9 [35].

MATERIALS AND METHODS

General Procedures

Bis-2-Aminoprop-1-yl PEG-1900, PEG-4000, PEG-6000, PEG-8000 potassium phthalimide, thioanisol, acryloyl chloride, sarcosin ethylester, MSNT, Dhbt-OH, and TBTU were obtained from Fluka; TEMED and sorbitane monolaurate (Span-20) from Sigma. Ammonium persulfate, hydrazine hydrate and 1,2-ethanedithiol were obtained from Riedel-de Haën and TFA from Merck. Y(NO_2)-OH, Abz-OH (Fluka) were transformed into Fmoc-Y(NO_2)-OH, Boc-Abz-ODhbt, respectively as previously described [36]. Fmoc-amino acid-OPfp esters and HMBA linker were purchased from Bachem. Acryloyl sarcosin ethyl ester was prepared by the reaction of acryloyl chloride and sarcosin ethyl ester as reported in literature [4]. All other commercially available analytical grade reagents were used without purification, except dichloromethane which was distilled from P_2O_5 and stored over 4 Å molecular sieves. Polymerization were performed in 250 or 850 ml laboratory-scale suspension polymerization vessels as previously described [31]. Peptide substrate libraries were synthesized by a wet mixing method in a 20 column MCPS system as described [27]. Soluble peptide substrates were synthesized using high functional group capacity PEGA resin in 20 column MCPS apparatus [27]. Analytical HPLC was performed using a Waters RCM 8×10 module and with a Deltapak C-18 column (19×300 mm^2). Preparative HPLC was performed on a Hitachi L-6250 Preparative Intelligent Pump using Delta Pak C-18 column (25×200 mm^2). The solvent system for both analytical and preparative HPLC was buffer A, 0.1% TFA in water, and buffer B, 0.1% TFA in 90% acetonitrile–10% water and UV detection was 215 or 280 nm. The gradient for analytical HPLC (1

ml/min): a linear gradient 0–100% buffer B over 50 min and preparative HPLC (10 ml/min): a linear gradient of 20–70% buffer B over 100 min unless otherwise indicated. Purified peptides were hydrolysed with 6N HCl at 110°C and subjected to amino acid analysis (Alpha Plus AA analyser, LKB). Sequence analysis (ABI model 477A or 470A protein sequencer) was performed on resin beads that were placed on filter. The substitution level of Fmoc–resin was determined by spectrophotometric analysis (absorption 290 nm) of fulvene–piperidine adduct formed upon deprotection of amino groups using a Perkin-Elmer Lambda 7 UV/VIS spectrophotometer. The values obtained were correlated with a standard curve. Enzyme kinetics were performed using a temperature-controlled Kontron SFM 25 fluorescent spectrofluorometer. All MALDI-TOF experiments were carried out on a Finnigan Lasermat 2000 using an ACH matrix.

Bis-Chloro-polyethylene Glycol$_{6000}$ (1). PEG_{6000} (5 g, 8.3 mmol) was melted by heating on an oil bath at 100°C followed by drop-wise addition of thionyl chloride (3.65 ml, 50 mmol) within 30 min. The reaction mixture was stirred at 100°C overnight, and then cooled to room temperature. The product was precipitated by slow addition of diethyl ether (200 ml) with rapid stirring. Stirring was continued for 15 min cooling the reaction mixture in an ice bath. The precipitate was filtered off, washed with ether and dissolved in CH_2Cl_2 (50 ml). The chloro-PEG was precipitated from the CH_2Cl_2 solution by addition of ether. The precipitate was filtered off, washed with ether and dried in vacuum to give **1** (46 g, 92%) δ^{13}C–NMR 250 MHz: ($CDCl_3$) 42.6 (Cl–$\underline{C}H_2$–CH_2–), 71.3 (Cl–CH_2–$\underline{C}H_2$–) For PEG: 61.7 (HO–$\underline{C}H_2$–CH_2–), 72.5 (HO–CH_2–$\underline{C}H_2$–).

Bis-Phthalimido-polyethylene Glycol$_{6000}$ (2). Bis-chloro-PEG (**1**, 22 g, 3.7 mmol) and potassium phthalimide (10.5 g, 56.7 mmol) was suspended in dry DMF (60 ml). The suspension was slowly heated to 50°C, tetradecyl trimethyl ammonium bromide (75 mg) was added and the mixture was headed to 100°C in an argon atmosphere for 4 h. The precipitate was filtered off the diethyl ether was added slowly to the clear filtrate with stirring. Stirring was continued for another 30 min in an ice bath after the precipitation was complete. The precipitate was filtered, washed with ether, and dissolved in CH_2Cl_2 (60 ml). The insoluble impurities were filtered off and the filtrate was concentrated. The PEG–phthalimide was then precipitated with ether and dried in vacuum to yield **2** (20 g, 90%) δ^{13}C-NMR 250 MHz: ($CDCl_3$) 37.1 (Pht–N–$\underline{C}H_2$–), 68.8 (N–CH2–$\underline{C}H_2$), 133.8 (Ar) 123.1 (Ar), 168.1 (C==0).

Bis-Amino-polyethylene Glycol$_{6000}$ (3). Bis-phthalimido-PEG (**2**, 41 g, 6.6 mmol) and hydrazine hydrate (20.5 ml, 414 mmol) in absolute alcohol (150 ml) was heated under reflux for 12 h. After cooling to room temperature, the insoluble impurities were filtered off and washed with CH_2Cl_2 and the filtrate concentrated. The product was precipitated by addition of ether while stirring on an ice bath. The precipitate was filtered and re-dissolved in CH_2Cl_2 (60 ml) and the insoluble impurities were removed by filtration. The filtrate was concentrated and ether added slowly to precipitate the product. The product was filtered off, washed and dried in vacuum to yield 36 g of **3** (90%). δ^{13}C-NMR 250 MHz: ($CDCl_3$) 41.8 (NH_2–$\underline{C}H_2$–CH_2–), 73.5 (NH_2–CH_2–$\underline{C}H_2$–).

Bis-amino-polyethylene glycol$_{4000}$ and bis-amino-polyethylene glycol$_{8000}$ were prepared as described above using the appropriate amount of PEG derivatives.

Synthesis of Partially Acryloylated PEG$_{1900,\ 4000,\ 6000\ or\ 8000}$ (4). $(Acr)_{0.77}$ PEG_{1900}, $(Acr)_1$ PEG_{4000}, $(Acr)_1$ PEG_{6000} and $(Acr)_{1.2}$ PEG_{8000} were prepared using the appropriate amount of acryloyl chloride essentially as described [31] (cf Table 1) and directly used in the polymerization reaction.

Synthesis of bis-Acrylamido PEG$_{1900,\ 4000,\ 6000\ or\ 8000}$ (11). Acryloyl chloride (2 equiv.) was added drop-wise to a mixture of bis-amino-$PEG_{1900,\ 4000,\ 6000\ or\ 8000}$ (1 equiv.) and triethylamine (2 equiv.) in CH_2Cl_2 stirred at 0°C. The reaction was continued at 0°C for 1 h and the salts were removed by filtration and worked up as described [31].

Synthesis of Acryloyl Sarcosin Ethyl Ester (4) (12). Sarcosin ethyl ester (1.00 g, 6.5 mmol) was dissolved in $CHCl_3$ (10 ml) and cooled with stirring in an ice-bath. Triethylamine (1.85 ml, 13 mmol) was added in 5 min followed by a solution of acryloyl chloride (0.53 ml, 6.5 mmol) in $CHCl_3$ (10 ml) during 30 min. The cooling bath was removed and the reaction mixture stirred over night. The precipitate was filtered off and filtrate evaporated to dryness. The residue was directly used in polymerization reactions.

Inverse Suspension Polymerization Reactions, Synthesis of Polymers 6–9 and 13–19. Polymerization reactions were carried out in a mixture of *n*-heptane–carbon tetrachloride (6:4 v/v, 138 or 470 ml)

Table 1 The Synthesis of Different Types of PEGA Supports

Monomers used (g)	Type **I** resin				Type **II** resin						
	6	**7**	**8**	**9**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
MW (g/mole)[a]	1900	4000	6000	8000	1900	1900	1900	1900	4000	6000	8000
PEG (g)	60	61	54	60.6	10.54	10.54	10.54	42.5	42	58	48
$(Acr)_n^b$	0.77	1.0	1.0	1.2	2	2	2	2	2	2	2
DMA	–	–	–	–	1.49	1.49	1.49	–	–	–	–
Acrylamide	10	5	2.99	2.6	–	–	–	–	–	–	–
ASEE	–	–	–	–	1.11	1.89	3.33	3.42	3.42	2.22	2.56
NH_2 cap. (mmol/g)	0.11	0.13	0.086	0.077	0.44	0.52	0.83	0.43	0.48	0.22	0.2
Yield (g)	40	38.5	28	30	10.5	11.8	12.33	38	30.5	50.7	32
% Yield	90	80	67	55	80	85	80	83	67	84	63
Scale (ml)	850	850	850	850	250	250	250	850	850	850	850

[a] MW is the average molecular weight of the PEG used.
[b] n is the no. of equiv. of acryloylchloride added.

in 250 or 850 ml polymerization flask fitted with an overhead stirrer (speed, 650 r.p.m.) maintained at 70°C under argon atmosphere. Monomers (Table 1) were dissolved in water (28 ml or 95 ml) and DMF was added if necessary and purged with argon (5 min). This was followed by addition of sorbitan monolaurate (180 or 300 mg) in DMF (730 μl or 2.5 ml) and ammonium persulphate (200 or 705 mg) in water (730 μl or 2.5 ml). The reaction mixture was stirred vigorously and transferred to the polymerization flask rapidly. After 2 min, tetramethyl ethylenediamine (TEMED) (600 μl or 2 ml) was added to the mixture and the sticky point was reached within 30 s. The reaction set up was maintained at 75°C with constant stirring for 2–3 h. The resin was cooled, filtered off and washed with ethanol (2 vol.) and water (3 vol.) and passed through standard sieves (200–300 mesh). The resin was transferred to the filter, washed with ethanol (3 vol.) and CH_2Cl_2 (3 vol.) and dried first under low vacuum (water pump, 1 h) and then on a lyophilizer for 30 h (Table 1).

Swelling Studies

A known weight of the resin was taken in a syringe fitted with a sintered Teflon filter and swelled in a given solvent for 15 min. The swelled resin was compressed with the piston of the syringe and the pressure was slowly released. The volume of resin at this point was noted and related to the sample weight to obtain the resin swelling abilities. The experiment was repeated to ensure reproducible values (Figures 1 and 2).

General Methods of Solid-phase Synthesis

The synthesis of peptides were carried out manually using a disposable plastic syringe fitted with a sintered Teflon filter (pore size 70 μm) and connected to a vacuum waste bottle through a two-way Teflon valve. Library synthesis and MCPS was performed in a 20 column Teflon block [27]. In general, peptide synthesis was carried out using fully protected N^{α}-Fmoc amino acid OPfp esters (3 equiv.) in DMF with the addition of Dhbt-OH (1 equiv.) as an acylation catalyst and an indicator of the end point of acylation reaction. The side-chain protecting groups were *O*-*t*Bu for Asp and Glu, *t*Bu for Tyr, Hyp, Ser and Thr, trityl for Asn and Gln, Boc for His, Lys and Trp and 2,2,5,7,8-pentamethyl chroman-6-sulphonyl (Pmc) for Arg. The Fmoc group was removed with 20% piperidine in DMF. After the library synthesis the resin was washed with CH_2Cl_2 (6 vol.) and dried with suction. The side-chain protecting groups were removed by repeated treatment with a mixture TFA:thioanisol:ethane dithiol:water (87.5:5:2:5:5% by volume, 10 min and 2.5 h). The resin was washed with 95% acetic acid (4 vol.); DMF (6 vol.); 5% DIPEA/DMF (2 vol.); DMF (6 vol.); CH_2CL_2 (6 vol.) and finally dried under vacuum. Peptides were coupled to the HMBA-linker were first treated with 95% acetic acid (4 vol.), DMF (4 vol.) neutralization of resin with DIPEA in DMF (2 vol.) and CH_2Cl_2 (6

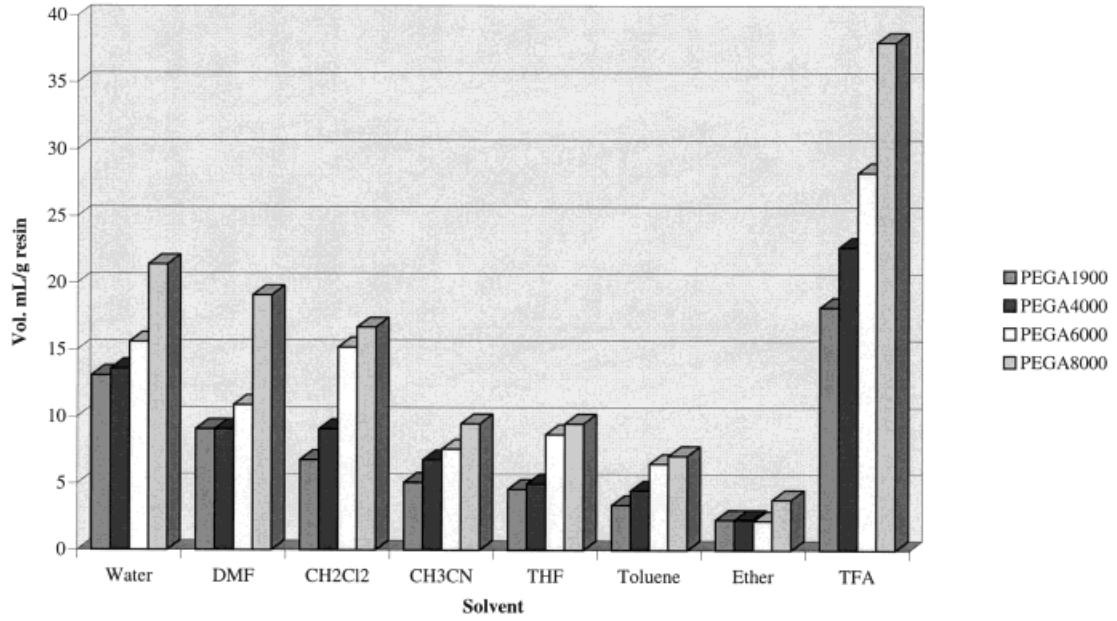


Figure 1 Comparison of swelling properties of PEGA Type I supports.

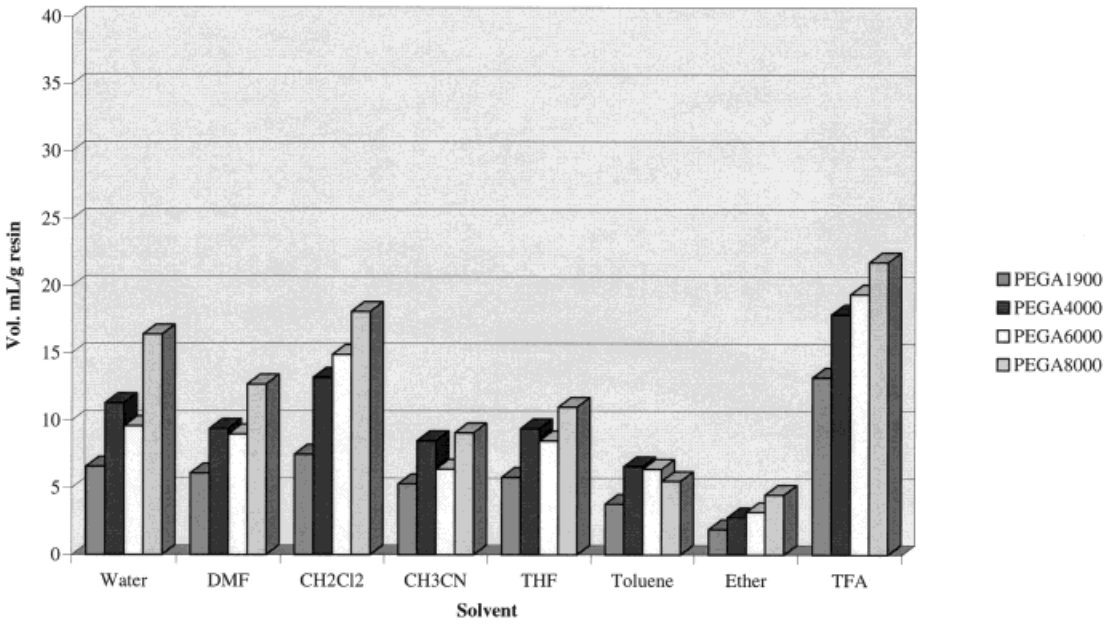


Figure 2 Comparison of swelling properties of PEGA Type II supports.

vol.). The resin was dried by suction for 1 h and then *in vacuo*. Cleavage from the resin was carried out by treatment with 0.1 M NaOH for 2 h and the resin washed with water (7 vol.). Finally, the filtrate was neutralized (pH paper) with 0.1N HCl and the crude peptide was purified by preparative HPLC.

Derivatization of PEGA Resin with HMBA Linker and MSNT Coupling of First Amino Acid

HMBA (3 equiv.) was dissolved in DMF followed by addition of TBTU (2.85 equiv.) and NEM (6 equiv.). After stirring for 3 min, the solution added to the PEGA resin (1 equiv. NH_2) which had been swelled

in DMF (1 h) in a plastic syringe. The reaction mixture was kept at room temperature for 4 h with gentle shaking. The resin was filtered, washed with DMF (6 vol.) and CH_2CL_2 (6 vol.) and dried on a lyophilizer for 1 day. *N*-Methyl imidazole (MeIm) (2.35 equiv.) was added to a solution of N^{α}-Fmoc amino acid (2.5 equiv.) in dry CH_2Cl_2 under argon. The resulting solution was added to a flask containing 1-mesityl-sulphonyl-3-nitro-1,2,4-trizole (MSNT) (2.5 equiv.). After 1 min the activated amino acid derivative was added to the HMBA–resin swelled in CH_2Cl_2. After 30 min the resin was filtered and the coupling reaction was repeated. The product resin was washed with CH_2Cl_2 (3 vol.), DMF (3 vol.), CH_2Cl_2 (3 vol.) and dried on a lyophilizer. The resin loading was quantified by measuring the UV-absorption of the benzfulvene/piperidine adduct formed by treatment of 20% piperidine/DMF and relating to a standard curve.

Synthesis of Y(NO_2)GPLGL-Nle-ARK(ABz)-PEGA_{4000}

$PEGA_{4000}$ resin (2 g, 0.13 mmol/g) was packed in a syringe, swelled in DMF and washed successively with 20% piperidine in DMF (25 ml), DMF (3×35 ml vol.) and excess solvent removed. A solution of Fmoc-Lys(Boc)-OH (450 mg 1.0 mmol) in DMF (25 ml) was activated with TBTU (300 mg 0.9 mmol) and NEM (0.15 ml, 1.1 mmol) and added to the resin. After 4 h the resin was washed with DMF (3 vol.) and CH_2Cl_2 (3 vol.) and treated with 50% TFA/CH_2Cl_2 (2 vol., 1 and 20 min). The resin was washed with CH_2Cl_2 (3 vol.) and DMF (3 vol.) and the free amino group of the lysine side chains was reacted with Boc-Abz-ODhbt (573 mg, 1.5 mmol) in the presence of a base, NEM (0.2 ml, 1.5 mmol). The resin was washed with DMF (3 vol.) and CH_2Cl_2 (3 vol.) after 1 h and dried under high vacuum.

A portion of this resin (200 mg, 0.026 mmol) was employed in the synthesis of the peptide substrate as described in the general methods. After the synthesis the resin was treated for 2 h with 95% aqueous TFA, washed with CH_2Cl_2 and DMF, neutralized with 10% DIPEA/DMF washed with DMF and CH_2Cl_2 and dried. A few beads were analysed by gas phase amino acid sequencing.

Table 2 Hydrolysis of Selected Substrates by Mouse MMP-9 and Subtilisin Carlsberg[a]

	K_M (μM)		k_{cat}/K_M (μM^{-1} min^{-1})	
Peptides	MMP-9	Subtilisin	MMP-9	Subtilisin
22	50.0	123.9	0.080	0.001
27	n.c.[c]	62.9	low[b]	3.8
35	94.9	48.9	1.2	64
42	262.2	197.0	1.0	0.085
43	143.1	811	0.32	1.9
44	326.3	43.7	0.16	2.4
45	n.c.	n.d.[d]	n.c.[c]	n.d.[d]
46	10.2	n.c.	0.018	n.c.
47	218.3	36.9	0.014	23
48	n.c.	n.d.	n.c.	n.d.
49	n.c.	n.d.	n.c.	n.d.
50	384.0	28.7	0.080	n.c.
51	80.1	189.3	0.10	4.4

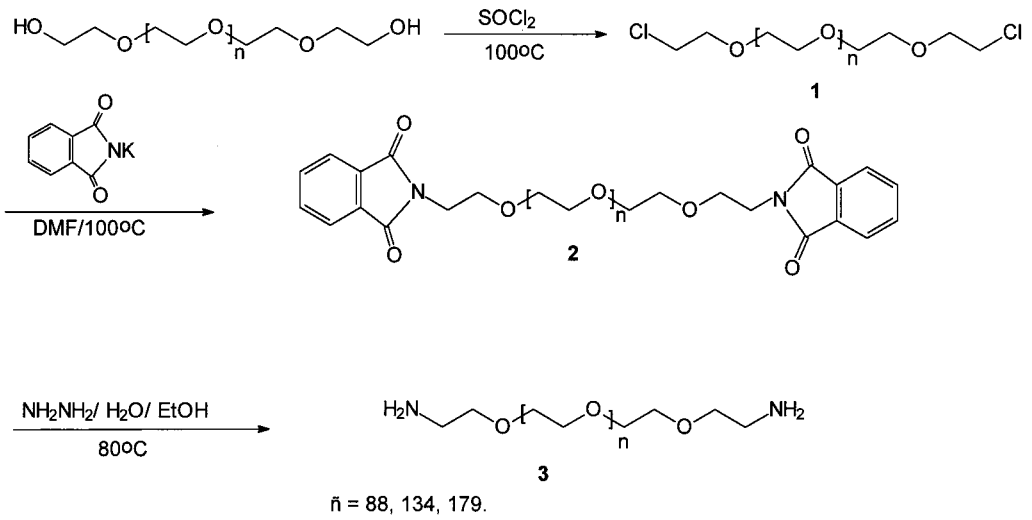
[a] Assays were performed at 37°C, pH 7.5.
[b] The cleavage of CL-6 by was clearly detectable, but k_{cat}/K_m was below the measurable level.
[c] n.c.: no cleavage could be detected even after two days of incubation.
[d] n.d.: not done.

Enzymatic Cleavage of PEGA-bound Model Substrate

A sample of the peptide resin was swelled in 2 ml buffer (10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35, 50 mM Tris–HCl, pH 7.5). The solvent was removed and MMP-9 enzyme in the same buffer was added. The reaction mixture was incubated at 37°C with shaking for 7 h. The enzyme reaction was stopped by washing the resin with 2% aqueous TFA, neutralizing with 10% $NaHCO_3$ solution and finally washing with deionized water.

Determination of k_{cat}/K_m for Substrates Incubated in Solution with MMP-9

The putative substrates were prepared as 10 mM stock solutions in dimethyl formamide (DMF). Fluorescence assays were performed at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 425$ nm. A typical assay was performed by incubating 126 μl of various concentrations of substrate with 14 μl of an enzyme solution at 37°C in the reaction buffer consisting of 50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.01 M $CaCl_2$, 0.05% Brij 35 to which was added DMF to a final concentration of 0.5% (v/v). The reaction was arrested by adding 840 μl of 3% (v/v) glacial acetic acid. The amount of substrate hydrolysis was calculated after subtraction of the reaction blank value (arresting solution added before incubation). The initial rates of hydrolysis were determined over a substrate concentration range of 0.5–60 μM using a final concentration of 8 nM rabbit osteoclast MMP-9 or 3 nM subtilisin Carlsberg, respectively (Table 2). The kinetic parameters (k_{cat} and K_m) were calculated assuming



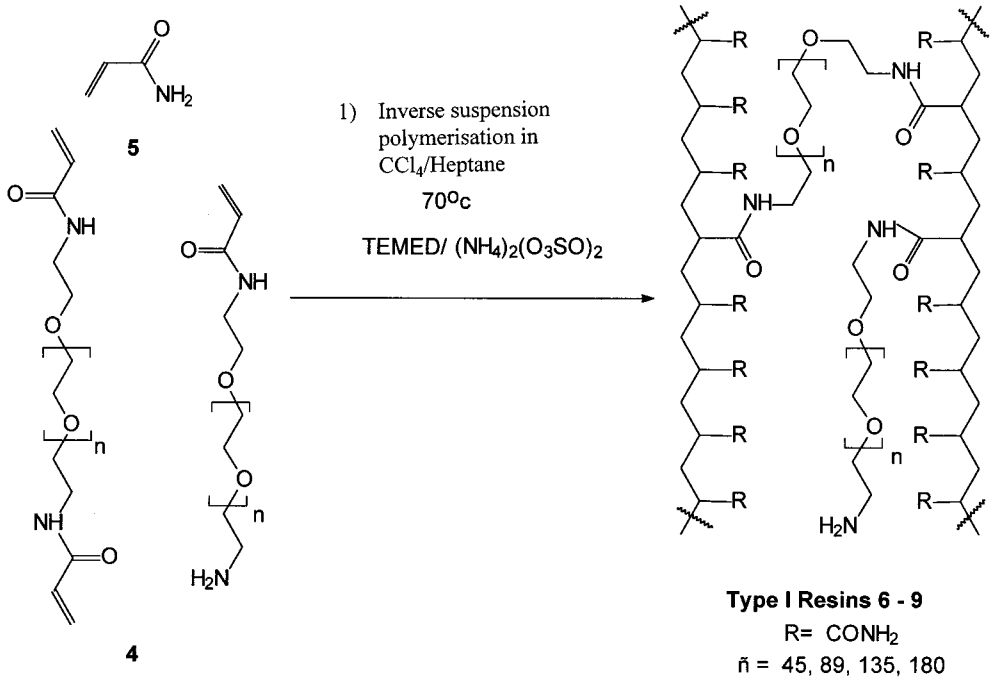
Scheme 1 Synthesis of bis-amino-$PEG_{4000,\ 6000\ and\ 8000}$.

Michaelis–Menten kinetics by using the Leonora software [37].

The latent proform of mouse MMP-9 originated from conditioned medium of a transfected Baby Hamster Kidney cell line [38] cultured in the absence of fetal calf serum for 1 day at 37°C. The latent proform of rabbit MMP-9 originated from the conditioned medium of an osteoclast preparation [39]. Briefly, the long bones were isolated from 10-day-old rabbits, depleted from marrow by aspiration and minced thoroughly with a pair of scissors. Cells were released from the bone particles by mechanical agitation and an osteoclast-rich preparation was isolated by centrifugation (30 *g*, 5 min) and seeded on Petri dishes for 90 min in the presence of 2% fetal calf serum. The non-adhering cells were removed by gentle washing and the remaining osteoclasts cultured under serum-free conditions in the presence of 40 nM phorbol 12-myristate 13-acetate. Pro-MMP-9 was purified from the conditioned medium on a hydroxyapatite column, activated from 24 h at 37°C by APMA (1 mM) in the reaction buffer and stored in aliquots at −80°C until use. As a general control the proteolytic enzyme subtilisin Carlsberg was used [27].

Synthesis of Peptides Using the High-Capacity Resin

Abz-AFRFAAAY(NO$_2$)PPD (20). Fmoc-Asp(tBu)-HMBA–Resin (0.24 g, 0.45 mmol/g) was used in the synthesis of peptides in a completely automated custom-made continuous flow peptide synthesizer. Step-wise synthesis was carried out using Fmoc-amino acid OPfp ester (3 equiv.) in presence of DhbtOH (0.1 equiv.) Fmoc-Y(NO_2) (0.103 g, 0.24 mmol) was coupled by TBTU (0.069 g 0.21 mmol) activation in presence of NEM (0.055 g, 0.48 mmol). The final coupling was carried out using Boc-Abz-ODhbt (0.039 g 0.24 mmol). After the synthesis the peptide resin was transferred into a syringe and washed with DMF (3 × 10 ml) and CH_2Cl_2 (3 × 10 ml) and dried in a lyophilizer. The dry resin was treated with 95% TFA–water mixture (1 × 6 ml, 1 min and 1 × 6 ml, 2.5 h) for the side-chain deprotection. The resin was washed with 95% acetic acid (3 × 10 ml), CH_2Cl_2 (3 × 10 ml), DMF (3 × 10 ml), 10% DIPEA in DMF (3 × 10 ml) and CH_2Cl_2 (3 × 10 ml) and dried in a lyophilizer to yield 370 mg peptide resin. The peptide resin (50 mg) was treated with 0.1N NaOH (1.5 ml) in a syringe. After 2 h at 20°C the resin was filtered off and filtrate and washings collected. The filtrate was neutralized (pH paper) by the addition of 0.1N HCl and the peptide purified on HPLC to yield 16.2 mg pure peptide (90%). Mass *m*/*z* 1390.6 ($C_{66}H_{84}N_{16}O_{18}$ required M + H = 1390.5). Interestingly, the MALDI-TOF-MS in α-cyano-4-hydroxycinnamic acid (ACH) matrix shows prominent peaks at 1375 and 1359 due to the photochemical reduction of Y(NO_2) in the ACH matrix under influence of the laser beam [40]. Bradykinine was used as the internal standard (M + H = 1061.2). An electron spray mass spectra of this peptide shows only one peak at 1390.1 (M + H) with no reduction, confirming the purity of the product. Amino acid analysis: Asp 0.94 (1); Ala 4.01 (4); Phe/Tyr(NO_2) 2.88 (3); Arg 0.85 (1); Pro 1.8 (2).



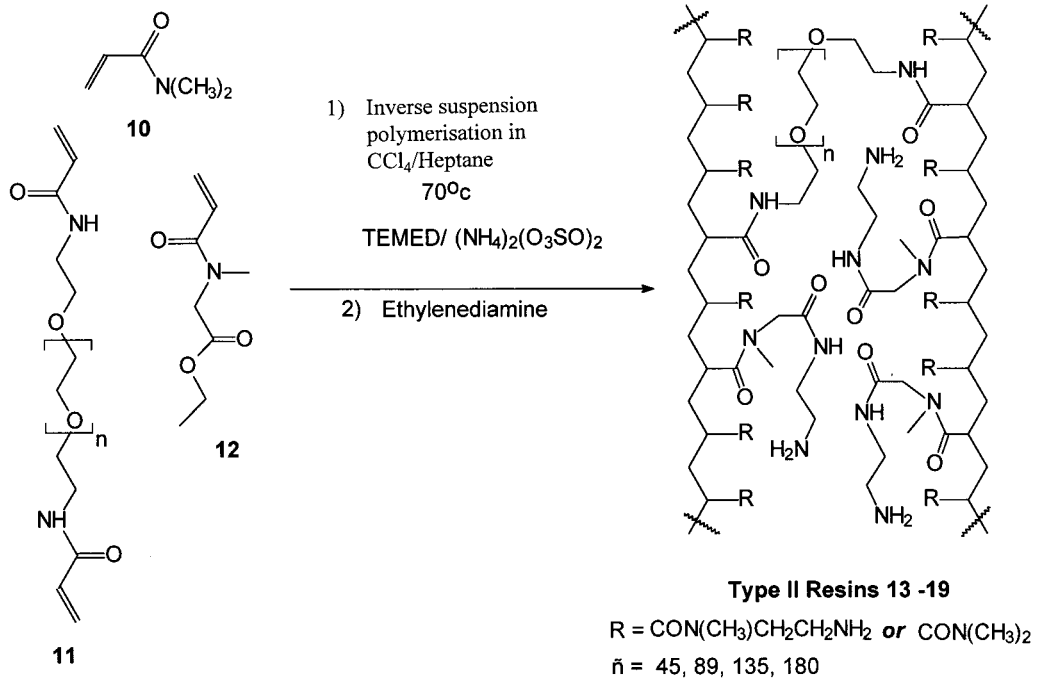
Scheme 2 Synthesis of long-chain less cross-linked PEGA polymer supports.

VITAFNEGLK (21). The peptide was synthesized using Fmoc-Lys-HMBA-Resin (0.26 g, 0.8 mmol/g) in an automated continuous flow peptide synthesizer as described above. After the synthesis, 400 mg of peptide resin was obtained. NaOH cleavage as above, yielded 89% crude peptide showing essentially one major peak by analytical HPLC, was purified by RP-HPLC affording (64% overall yield). Mass: *m/z* 1092.3 ($C_{50}H_{82}N_{12}O_{15}$ required $M+H=1092.4$). Amino acid analysis: Val 0.54 (1); Thr 1.03 (1); Ala 1.03 (1); Phe 0.97 (1); Asn 1.00 (1); Glu 0.99 (1); Gly 1.03 (1); Leu 0.96 (1); Lys 0.97 (1); Ile 0.51 (1). The low values in amino acid analysis for Ile and Val were due to partial hydrolysis of the Val-Ile bond.

RESULTS AND DISCUSSION

Synthesis of Improved PEGA Supports

In a previous study it was demonstrated that enzymes up to 50 kDa can enter the $PEGA_{1900}$ polymer matrix [31,41]. In order to develop selective enzyme inhibitors for MMP-9 it became necessary to increase the apparent pore size of PEGA supports since MMP-9 which in its proform has a MW of 92 kDa, even after cleavage of its propeptide by activation, exist in forms of approx. 67/83 kDa [42]. This led to the development of long chain cross-linked $PEGA_{4000-8000}$. $PEGA_{1900}$ was obtained by the copolymerization of partially acryloylated commercially available bis-amino-PEG_{1900} and acrylamide [31]. For making long chain cross-linked PEGA supports bis-amino-$PEG_{4000,\ 6000,\ 8000}$ were synthesized by a three-step reaction starting from PEG-OH according to the reported procedure [31] with slight modifications. First PEG-OH was converted to bis-chloro-PEG **1** by reflux with thionyl chloride (Scheme 1). The product bis-chloro-PEG was converted to bis-phthalimido-PEG **2** by treatment with potassium phthalimide and finally bis-amino-PEG, **3** was obtained by hydrazinolysis of the phthalimide-protecting group. All the reactions were followed by ^{13}C-NMR (Figure 1). The bis-amino–PEG was reacted with acryloylchloride to form partially or bis *N*-acryloylated PEG and the product was used as macromonomer in an inverse suspension polymerization reaction. Type **I** resins **6–9**, are crosslinked to a limited extend ($<5\%$) based on weight of the polymer. In these polymers partially acryloylated bis-amino–$PEG_{1900,\ 4000,\ 6000\ or\ 8000}$ were copolymerized with acrylamide (Scheme 2 and Table 1). The resins have high swelling capacities and are suited for enzyme reactions. However, they have comparatively low amino group capacities of 0.13–0.08 mmol/g. Another modification carried out in the present study was to increase the amino



Scheme 3 Synthesis of long-chain more cross-linked high-capacity PEGA polymer supports using sarcosine ethyl ester.

group capacity of the PEGA support by introducing sarcosin ethylester [4] as a functional group monomer in the polymerization mixture. Inverse suspension copolymerization of acryloyl sarcosin ethyl ester **12** (prepared by reaction of acryloyl chloride with sarcosin ethyl ester hydrochloride in the presence of triethyl amine) with bis-acrylamido-$PEG_{1900,\ 4000,\ 6000,\ 8000}$ resulted in a PEGA *co*polymer Type **II** (resins **13–19**) with a loading ranging from 0.22–1.0 mmol/g. *N*,*N*-Dimethyl acrylamide **10** was added in certain cases (Scheme 3). The synthesis of the polymeric support was performed with crystalline bis-acrylamido-PEG. It was comparatively easy and gave a high yield of PEG incorporation (90–100%). Type **II** resins **9–13** are more highly cross-linked, where bis-acrylamido $PEG_{1900,\ 4000,\ 6000\ or\ 8000}$ were copolymerized with sarcosin ethyl ester. The bead sizes of the different polymers were measured under a stereo microscope by comparing with standards.

Synthesis of Solid-phase Substrate Libraries and Their Use in Solid-phase Assays of MMP9

Fluorescent quenched peptide substrate libraries bound to PEGA resin have been effectively used in the identification of enzyme substrates [27]. In the present study, the technique has been applied for determination of the enzyme substrate specificity of MMP-9 isolated from osteoclasts [43]. Proteolytic enzymes such as MMP-9 and cathepsins seems to play a crucial role in osteoclast migration and bone resorption, but the specific roles of the individual proteinases are not known. The present work describes the successful development of a new highly permeable PEGA beaded support to facilitate mass transport of large enzymes such as MMP-9 (active forms: 67–83 kDa). The fluorescent quenched peptide library was synthesized on the new polymer using a multiple column library synthesizer [44] and was incubated with the enzyme (MMP-9). Thus, a peptide library $XXY(NO_2)XXXXXXK(Abz)PPPM-PEGA_{6000}$ was synthesized on low cross-linked Type **I** resin (0.09 mmol/g NH_2 group), where Abz and $Y(NO_2)$ are the fluorescent donor–acceptor pair. The X represents the 20 genetically encoded amino acids except Cys, which was replaced by Hyp, an important amino acid constituent of bone matrix proteins. The library was synthesized using Fmoc-amino acid–OPfp esters in the presence of DhbtOH by the portion mixing technique [27]. After the synthesis, the peptide library was fully deprotected by TFA treatment, neutralized and viewed under a fluorescence microscope, where several false positive beads were manually removed. The peptide library was incubated with the APMA-activated enzyme preparation at 37°C for about 40 h in the

Table 3 Multiple Column Peptide Synthesis (MCPS) of Putative Fluorogenic Substrates from First Library

Compound No.	Peptide substrates	Mol. Wt.	
		Calculated	Found M+H
22	AbzSKYP-Hyp-A↓LFY(NO_2)D(All)	1420.6	1421.3
23	AbzSRYEP-Hyp-G↓LTY(NO_2)D(All)	1516.7	1517.2
24	AbzGYEA-Hyp-G↓FTY (NO_2)D(All)	1339.4	1340.6
25	AbzTDY-Hyp-FN↓FTIY (NO_2)D(All)	1615.7	1616.6
26	TDY(NO_2)-↓Hyp-FNFTIK(Abz)D(All)	1580.7	1581.9
27	SKY(NO_2)P-Hyp-A↓LFFK(Abz)D(All)	1532.7	1533.4
28	SRY(NO_2)EP-Hyp-G↓LTK(Abz)D(All)	1482.6	1483.4
29	GY(NO_2)EA-Hyp-G↓FTK(Abz)D(All)	1264.3	1265.9
30	AIY(NO_2)AGM↓ITLK(Abz)D(All)	1399.6	1400.8
31	AbzAIYAG↓MITLY(NO_2)D(All)	1434.7	1435.5
32	SKY(NO_2)P-Hyp-Q↓LFFK(Abz)D(All)	1589.8	1590.2
33	SKY(NO_2)P-Hyp-F↓LFFK(Abz)D(All)	1608.8	1609.2
34	SKY(NO_2)PAA↓LFFK(Abz)D(All)	1516.7	1517.9
35	SKY(NO_2)PAA↓LFFK(Abz)D(All)	1490.7	1491.8
36	SKY(NO_2)P-Hyp-A↓AFFK(Abz)D(All)	1490.7	1491.3
37	SKY(NO_2)P-Hyp-G↓LFFK(Abz)D(All)	1518.7	1518.9
38	SEY(NO_2)P-Hyp-A↓LFFK9Abz)D(All)	1533.7	1533.9
39	SKY(NO_2)A-Hyp-A↓LFFK(Abz)D(All)	1506.7	1507.2
40	Y(NO_2)SKKP-Hyp-A↓LFFK(Abz)D(All)	1660.9	1662.1
41	SKY(NO_2)P-Hyp-↓ALTFK(Abz)D(All)	1486.7	1487.3

↓ Cleavage point

initial experiment. The bright beads were isolated manually under the fluorescence microscope and sequenced to identify peptide substrates for MMP-9. Ten potential peptide substrates were obtained in the initial screening procedure and were used as basis for design of substrates to be synthesized in a MCPS apparatus (Table 3). None of these peptides substrates, proved to be very good substrates for MMP-9 in solution according to a fluorescence quenched assay (Table 2) since the rates of enzymatic cleavage of these fluorescence quenched peptide substrates were slow when compared with a commercial standard substrate. However, according to k_{cat}/k_m values they were much more specific for MMP-9 when compared with subtilisin Carlsberg. The slow hydrolysis may be caused by the low concentration of the initial enzyme preparation leading to a long period of incubation (40 h) of the library to obtain fluorescent beads.

In order to optimize the enzymatic cleavage by the relatively large MMP-9 enzyme of subsequent polymer-bound peptide libraries a peptide substrate, Y(NO_2)GPLGL-Nle-ARK(Abz)-PEGA, for MMP was synthesized on $PEGA_{4000}$ beads and incubated with APMA-activated MMP-9 at 37°C. The cleavage reaction was monitored under a fluorescence microscope. The beads started to illuminate within 2 h and $< 10\%$ cleavage had occurred within 7 h as revealed by Edman-degradation sequence analysis. The loading capacity of the resin should also be high enough to facilitate sequence analysis. It was reasoned that by reducing the distance between the Abz/Y(NO_2) pair, a problem of false positive beads should be solved. Taking into account the above factors a second peptide library was synthesized on $PEGA_{4000}$ beads (0.13 mmol NH_2/g) with four amino acids instead of six between the donor–acceptor pair XXY(NO_2)-XXXXK(Abz)–$PEGA_{4000}$. Upon synthesis the library contained a few false positive beads, which were manually removed and sequenced. A Trp residue was found in between the donor–acceptor pair in almost all of them. The interference of Trp with the energy transfer may therefore be a reason for the occurrence of non-quenched beads in the library. It is possible that specific conformational constraints of the relative orientation of the chromophores can result in non-quenched beads. The peptide library was incubated with the enzyme for less time (7 h), the reaction was stopped and fluorescent beads were collected for

Table 4 Multiple Column Peptide Synthesis (MCPS) of Putative Fluorogenic Substrates from Second Library

Compound No.	Peptide substrates	Mol. Wt	
		Calculated	Found M+H
42	Y(NO_2)PL-Hyp-↓MKGK(Abz)G	1169.5	1170.8
43	FAY(NO_2)Hyp-↓MRAK(Abz)G	1219.5	1220.3
44	Y(NO_2)P-Hyp-M↓MRGK(Abz)G	1215.5	1216.7
45	ARY(NO_2)PKK↓VK(Abz)G	1209.7	1210.9
46	N-Hyp-Y(NO_2)P-Hyp-Hyp-↓YK(Abz)G	1243.5	1244.2
47	FAY(NO_2)-Hyp-↓MKM-Hyp-K(Abz)G	1364.6	1365.8
48	N-Hyp-Y(NO_2)P-Hyp-Hyp-↓MK(Abz)M-Hyp-G	1455.6	1456.7
49	Y(NO_2)P-Hyp-M↓MK(Abz)G-Hyp-G	1172.5	1173.4
50	Y(NO_2)P-Hyp-Hyp-↓MK(Abz)G-Hyp-G	1154.5	1155.8
51	FAY(NO_2)-Hyp-↓LK(Abz)G-Hyp-G	1144.5	1145.6

↓ Cleavage point.

analysis. The beads were sequenced to obtain 11 peptide sequences and used as a basis for design and synthesis of ten fluorogenic peptide substrates (Table 4). k_{cat}/K_m values showed that some of these were comparatively good substrates for MMP-9 (Table 2). However, the very high specificity observed in the solid phase was not observed in solution. This may be due to the high local concentration of substrate on the solid-phase facilitating cleavage of substrate with lower affinity for MMP-9, i.e. with high K_m values. Determination of the K_m values showed that the good substrates had indeed higher values (Table 2). Highly selective MMP-9 substrates **42, 46** and **50** were identified. The specificity of the assay showed high selectivity for a hydrophobic residue in P4, a Pro in P3, Hyp in P1, M or L in P1′, P2′ favours a basic residue such as R or K, G is best accepted in the P3′ position.

Peptide Synthesis Using More Cross-linked High-capacity Resins

The new, more highly cross-linked Type **II** supports **13–19** has similar physical and mechanical properties as that of a previously described PEGA support [31] and both the functional ester and the subsequently formed amino groups had sufficiently high reactivities for SPPS. The resin was evaluated for peptide synthesis in a custom-made fully automated continuous flow peptide synthesizer [40]. First, the resin was treated with ethylene diamine and the free amino group capacity was determined by incorporating Fmoc-Gly (0.4 mmol/g, **13** and 0.8 mmol/g, **15**) into the support and measuring the UV-absorption of the benzfulvene/piperidine adduct formed by treatment with 20% piperidine/DMF. The 4-hydroxymethyl benzoic acid linker was quantitatively attached to the amino functionalized resin by the TBTU procedure [45] using 2 equiv. acylating reagent. The first amino acid was coupled to the linker by the MSNT method [46]. The peptide, Abz-AFRFAAAY(NO_2)PPD-OH **20** was synthesized following standard SPPS methodology using the resin **13**. A 2.5-fold excess Fmoc-amino acid–OPfp esters along with Dhbt-OH was used for coupling reaction (time 2 h). After the synthesis the resin was washed with DMF and lyophilized. The resin showed considerable weight gain corresponding to 98% of the 11-residue peptide. The peptide resin was treated with 95% aq. TFA to remove all the side-chain protecting groups and the resin was washed, treated with DMF, 20% piperidine, washed with DMF and dichloromethane and lyophilized. A portion of this resin was treated with dilute aq. NaOH to cleave the free peptide from the support. The filtrate was collected and neutralized with aq. HCl and the purity determined on an analytical RP-HPLC column. After lyophilization it was found to be $>95\%$ pure (Figure 3), further purification was carried out on a preparatory RP-HPLC column to yield the pure peptide (80%). The pure peptide was characterized by analytical HPLC, matrix assisted laser-desorption mass spectrometry (MALDI-MS) and amino acid analysis. Similarly the resin **15** was reacted for 2 days with ethylene diamine, the capacity was determined and haemoglobin derived peptide H-VITAFNEGLK-OH was synthesized (89% crude

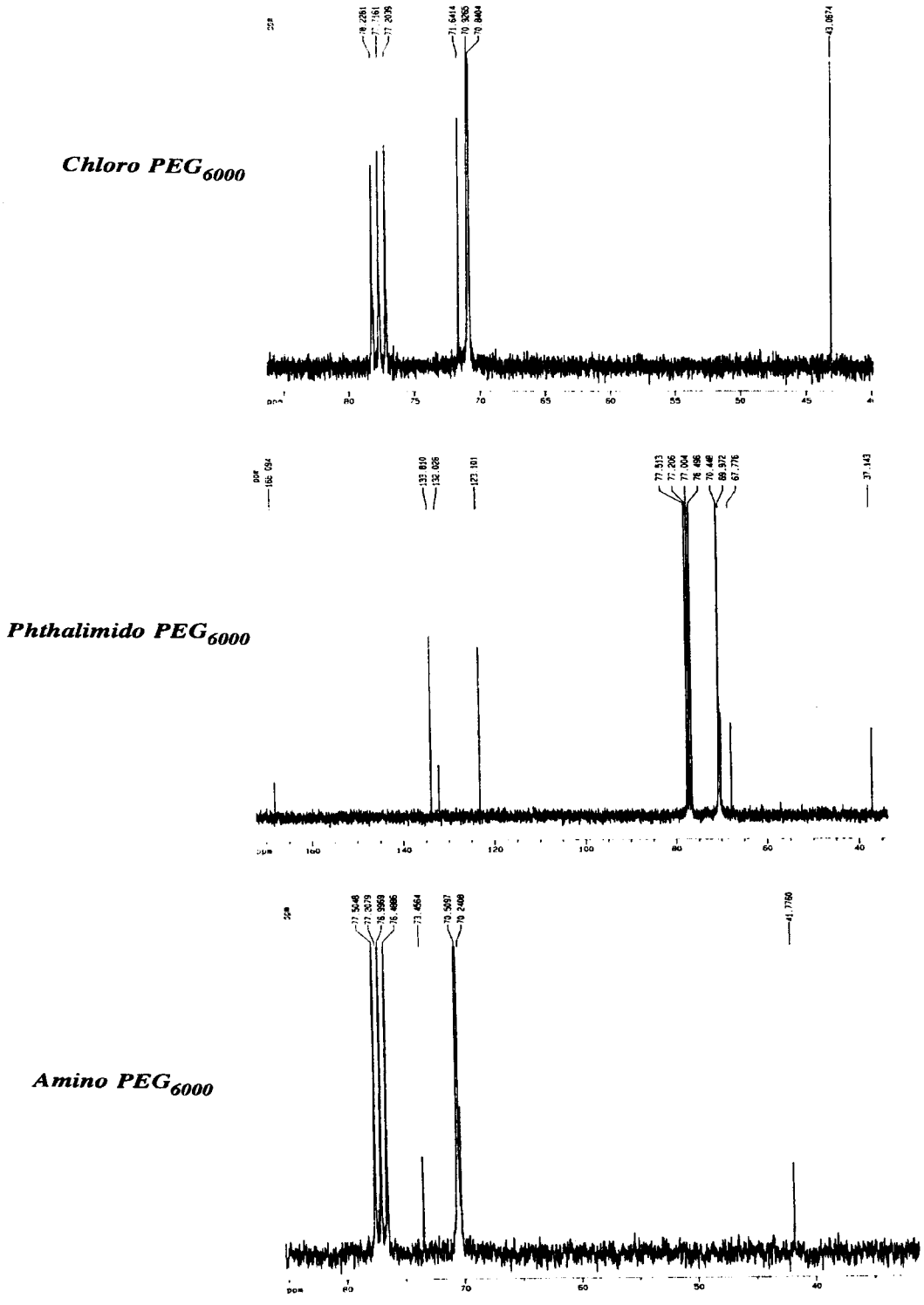


Figure 3 ^{13}C-NMR spectra indicating the purity of crude bis-chloro, phtpalimido and amino PEG_{6000}

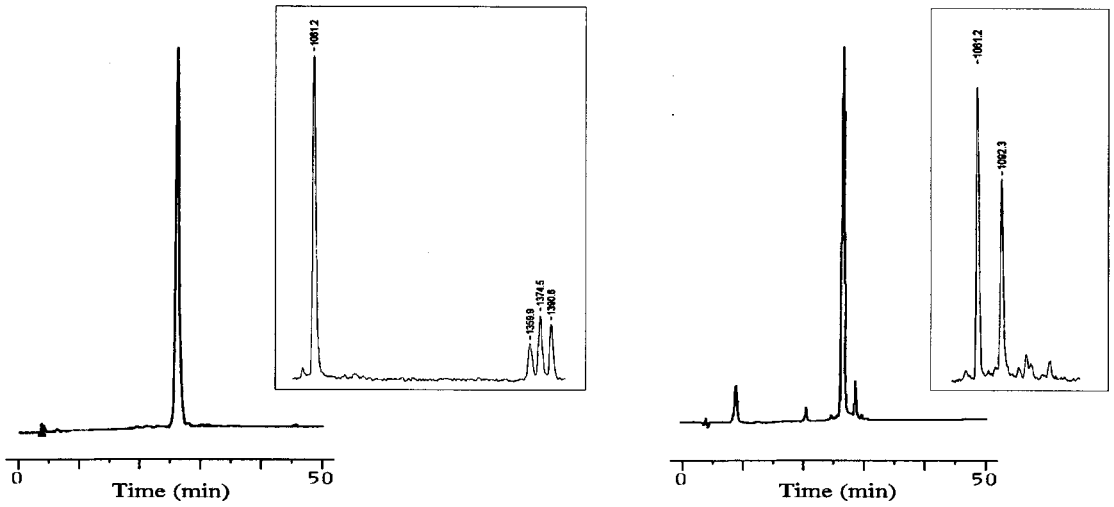


Figure 4 HPLC profiles of crude (left, A) fluorescence quenched peptide substrate Abz-AFRFAAAY(NO_2)PPD and (right, B) haemoglobin-derived peptide H-VITAFNEGLK-OH. The inset shows mass spectra of purified peptides (A) and (B) respectively.

yield), purified and characterized as described above (Figure 4). The mass of the peak at 29 min corresponded to loss of H_2O and the peak at 20 min was not peptide.

Swelling Studies of Various PEGA Supports

Maximum swelling of the resins were observed in polar solvents such as TFA, water followed by DMF and dichloromethane. This is due to the compatibility of the resin with these solvents. POE has a unique solubility in that it dissolves in water and dichloromethane to any extent. This has been explained using molecular dynamics calculations [47]. The swelling properties of the resins changes with the degree of cross-linking as evidenced in the Type **I** and Type **II** resins (Figure 1 and Figure 2). In general Type **I** resins have higher swelling capacity than Type **II** resins. This can be explained on the basis of cross-link densities. Type **II** are more highly cross-linked than Type **I**. Another significant observation is that the swelling of the resins (Types **I** and **II**) increases with the increase in PEG chain length. Owing to the high degree of solvation of PEG, the porous gel matrix is completely filled with solvent leading to an extended PEG cross-linked structure and a firm mechanically robust appearance of the swelled resin in solvents showing the most favourable swelling properties. Effective permeation of the resin matrix occurs under the influence of a swelling solvent leading to good exposure of reactive group for peptide bond formation or enzymatic cleavage. There is strong evidence from Raman [48] infrared spectroscopy and from molecular dynamics simulation [47] that POE forms a helix in aqueous solution. There are three possible helical conformations of PEG having the low-energy *gauche* conformation of vicinal oxygen–carbon bond. One has large hydrophobic turns with the oxygen atom in the interior of the helix, the second is a helix of intermediate polarity and third is a quite hydrophilic helix exposing the oxygen atom to the environment. The PEG molecule exists as a hybrid of these structures and has an amphipatic nature [47]. It is solvated well, both by polar and non-polar solvents. The ability of the PEG molecule of these resins to swell in various polar and non-polar solvents is a measure of the thermodynamic structural variation [47] and hydrophilic/hydrophobic balance. The exact swelling behaviour can be modulated depending on the detailed molecular composition of the resin with respect to amount and length of cross-linker and nature of added acrylic monomers.

CONCLUSION

A new class of high molecular weight PEG-based polymer supports was developed to facilitate easy penetration of large bio-molecules into the macromolecular matrix. A new method was developed for the large-scale production of pure bis-amino-$PEG_{4000-8000}$ involving only precipitation to afford

quantitative conversion of hydroxyl to amino function. The reaction were conveniently followed by ^{13}C-NMR. These resins have excellent swelling properties in both aqueous medium as well as organic solvents. It was found that the new resins can be efficiently used in synthesis of fluorescent quenched peptide libraries. Using the APMA activated 67–83 kDa forms of MMP-9 from ostoclasts, solid-phase enzymatic cleavage reaction conditions were optimized. From the library selective MMP-9 peptide substrates have been identified. Furthermore, preparation of high-capacity PEGA resins with well-defined loading of functional groups has been achieved. The preparation of monomers, polymerization, functionalization and work-up procedure of the resin is easy and the resin is economically competitive owing to the low price of the PEG. The physicochemical properties of the supports were found mainly to be determined by the flexible PEG polymer constituting 80–95% of the polymer weight. The resins allowed the preparation of peptides in high yield and purity. The dynamic gel-like properties and porous nature of PEGA resins allows fast mass transfer. Yet they are completely flow-stable, and have a high and uniform functional group reactivity facilitating completion of chemical or even enzymatic reactions. The highly solvated, flexible and amphiphilic PEG chains increase the solvation and accessibility of the growing peptide chain even at high peptide concentration. In order to obtain increased functional group capacity, the polyethylene glycol arm spacing the growing peptide chain from the polymer backbone in conventional PEGA–resins has been sacrificed. However, the effect of the shorter spacing arm on the peptide synthesis was marginal.

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