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Synthesis, Characterization and Biocompatibility of PEGA Resins

FRANCE-ISABELLE AUZANNEAU, MORTEN MELDAL* and KLAUS BOCK

Department of Chemistry, Carlsberg Laboratory, Valby-Copenhagen, Denmark

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Abstract: Three types of beaded polyethylene glycol polyacrylamide copolymers (PEGA) with a high content of polyethylene glycol (PEG) were synthesized by inverse suspension polymerization and characterized for peptide synthesis and with respect to their physical properties. Several peptides of high purity have been synthesized on the resin. The properties which were determined were loading of amino groups, swelling, bead size distribution, porosity, flexibility and compatibility with active biomolecules. A loading of 0.35 mmol/g has been obtained and the swelling was excellent in solvents of various polarities ranging from water to dichloromethane. The ^{13}C-NMR T_1-relaxation times of a resin containing a peptide were determined in DMSO-d_6 and the resin was found to exhibit a behaviour similar to the components in free solution.

Keywords: Synthesis resin; beaded PEG polymer; radical polymerization; solid-phase peptide synthesis; peptide library enzyme assay

Abbreviations

ABz, 2-aminobenzoyl; Acr_2PEG, bis-2-acrylamidoprop-1-yl polyethylene glycol; Acr_2PPG, bis-2-acrylamidoprop-1-yl polypropylene glycol; DBU, 1,7-diaza-(5,4,0)-bicycloundec-1,8-ene; DMA, *N,N*-dimethyl acrylamide; MCPS, multiple column peptide synthesis; $(NH_2)_2$PEG, *bis*-2-aminoprop-1-yl polyethylene glycol; $(NH_2)_2$PPG, *bis*-2-aminoprop-1-yl polypropylene glycol; PEG, polyethylene glycol; PEGA, polyethylene glycol polyacrylamide copolymers; PPG, polypropylene glycol; TEMED, tetramethyl ethylenediamine.

* Address for correspondence: Morten Meldal, Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby-Copenhagen, Denmark.

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INTRODUCTION

Since solid-phase peptide synthesis (SPPS) was first described by Merrifield in the early 1960 [1, 2], this technique has been widely applied to the preparation of peptides and also recently to glycopeptides [3–5]. The outcome of a multistep solid-phase synthesis is dependent on the structure of the peptide to be prepared, the strategy followed (amino acid activation, protecting groups, deprotection schemes), as well as the type of solid support employed [6–9]. Resins used in continuous flow SPPS must be stable towards the high pressures generated with high flow rates yet have an open structure which facilitates fast mass transfer [10–13]. In order to ensure a proper solvation of the reactive species allowing fast and quantitative reactions, their polarity must also be compatible with those of the reagents and solvents used, as well as with these of the resin-bound

growing peptide. Merrifield introduced the non-polar polystyrene resin, which has evolved into a 1% cross-linked beaded resin, still used in batch synthesis today [2]. A more polar dimethyl acrylamide resin has been developed in Sheppard's laboratory [7, 14]. However, neither of these are flow-stable under continuous flow conditions and often collapse after a few synthesis cycles. As it has been established that enhancing the stability of the resin by increasing the cross-linking did not lead to useful properties for peptide synthesis, the polyamide support was made flow-stable by polymerization of soft polydimethyl acrylamide gel inside a heterogeneous matrix of supporting kieselguhr [10]. Later, the irregular kieselguhr was replaced with a rigid, more regular, 50% cross-linked polystyrene sponge containing a polydimethyl acrylamide gel [13]. The 1% cross-linked polystyrene resin was also made both flow-stable and more polar by grafting polyethylene glycol as spacer for the peptide assembly [11, 12]. Other flow-stable materials, e.g. natural polymers [15] or modified polymer films [16, 17] have also been used.

In addition to organic synthesis, solid supports have also been widely used for biochemical applications. Thus, a number of techniques have been developed in which biomolecules were assayed for their binding properties [18, 19] or enzymatic activities [20, 21] using immobilized ligands bound to a polymer surface or bead. Such methods require complete biocompatibility of the polymer with the proteins involved, in order to avoid unspecific binding or even denaturation as frequently observed on polystyrene surfaces [22]. Composite materials have been developed to avoid these problems [22, 23] but even though polystyrene-based resins have been used successfully in several cases for binding studies, their permeabilities to biomolecules are restricted [24] probably due to the non-polar character of the polystyrene backbone.

In order to meet these requirements of both the peptide synthesis in organic solvents and biochemical assays in aqueous buffers a highly polar and flow stable solid support has been developed [8, 25]. Its major component (70–80%) di-acryloylated *bis*-aminopropyl-polyethylene glycol ($(NH_2)_2$PEG) was copolymerized with mono-acryloylated bis-aminopropyl–polypropylene glycol ($(NH_2)_2$PPG) (5–10%) in a polyamide resin to form a highly branched polymer network (PEGA resin). Because of the strong solvation properties of polyethylene glycol, this support is completely stable even with high flow rates and allows a rapid diffusion of the reagents throughout the polymer network. These properties rendered the PEGA resin very well suited for a number of peptide and glycopeptide syntheses [25–30] during which the coupling reactions could be followed by spectrophotometric monitoring of coloured resin-bound reagents, while the resin as such is transparent with no absorbance in the aromatic region of the UV spectrum (250–600 nm). Furthermore, the large swelling capacity of the PEGA resin makes it an attractive support for the preparation of longer peptides and the solvation effect of the PEG chains also allows the preparation of peptides which aggregate during the synthesis [8, 20]. The compatibility of proteins with PEG chains [31] and the permeability of the PEGA polymer network make these resins suitable for biochemical assays. In the present work, we report the preparation and characterization of various PEGA resins and demonstrate the large range of PEGA resins that can be easily prepared simply by varying the monomer composition.

MATERIALS AND METHODS

General Procedures

Commercially available analytical grade reagents were used without purification, except for *N,N*-dimethyl formamide (DMF) which was purified by fractional distillation at reduced pressure on a column of Rashig rings and dichloromethane which was distilled from P_2O_5 and stored over 4 Å molecular sieves. bis-2-Aminoprop-1-yl polyethylene glycol and acryloyl chloride were purchased from Fluka and amino acid derivatives from Bachem or MilliGen. Diethyl ether was obtained free of peroxides by passing through a column of alumina. It was either stabilized with hydroquinone (50 mg/l) or used immediately. Polymerizations were performed in a 250 or 850 ml laboratory-scale suspension polymerization apparatus [32] equipped with a four-blade (2 × 2) stirrer. To obtain the optimal stirring motion, the blades were arranged tilted and in a parallel fashion on each side of the stirrer rod. Resin samples swelled in water or DMF were examined with an American Optical Stereo Star fluorescence microscope equipped with a camera, and in order to increase the contrast, a few drops of a

dilute solution of bromophenol blue in DMF were added to the sample.

Synthesis of bis-Acrylamidopropyl Polyethylene Glycol 1900(3), $(Acr)_2PEG_{1900}$

Acryloyl chloride (24 ml, 2 equiv.) was added dropwise to a mixture of $(NH_2)_2PEG_{1900}$ (300 g, 150 mmol) and triethylamine (42 ml) in CH_2Cl_2 (370 ml), stirred at 0°C. When the addition was complete, the reaction mixture was stirred for 1 h at 0°C and the salts were removed by filtration and washed with CH_2Cl_2 (2 × 50 ml). The combined filtrate and washings were concentrated to dryness, hydroquinone-stabilized Et_2O (700 ml) was added to the residue and the product was allowed to crystallize for 5 h at 0°C under mechanical stirring. The crude product was filtered off, washed with stabilized Et_2O and dried (276 g, 87%). It was dissolved in CH_2Cl_2, filtered, concentrated and crystallized from peroxide free, unstabilized Et_2O as described above. $(Acr)_2PEG_{1900}$ was obtained pure as a white powder (245.6 g, 78%) that was stored at −20°C.

Synthesis of Partially Acryloylated $(NH_2)_2PEG_{1900}$ (4), $(Acr)_NPEG_{1900}$

$(Acr)_{0.25}PEG_{1900}$, $(Acr)_{0.5}PEG_{1900}$ and $(Acr)_{1.1}PEG_{1900}$ were prepared to be used directly in the 250 ml polymerization apparatus. Three solutions of acryloyl chloride (175 µl, 0.25 equiv.; 350 µl, 0.50 equiv.; 760 µl, 1.1 equiv.) in CH_2Cl_2 (respectively, 3, 6 and 13 ml) were each added dropwise to three separate solutions of $(NH_2)_2PEG_{1900}$ (17.06 g, 8.53 mmol) in CH_2Cl_2 (12 ml) that were stirred at 0°C. When the additions were complete, the reaction mixtures were stirred for 1 h at 20°C and stored at 4°C. Concentration and drying *in vacuo* at 20°C gave the partially acryloylated compounds $(Acr)_{0.25}PEG_{1900}$, $(Acr)_{0.5}PEG_{1900}$ and $(Acr)_{1.1}PEG_{1900}$ as opalescent, colourless, thick oils.

$(Acr)_{0.77}PEG_{1900}$ was prepared in a larger scale to be used directly in the 850 ml polymerization apparatus. A solution of acryloyl chloride (1.8 ml, 0.77 equiv.) in CH_2Cl_2 (30 ml) was added dropwise to a solution of PEG_{1900} (58 g, 29 mmol) in CH_2Cl_2 (40 ml) stirred at 0°C. After 1 h at 20°C concentration of the reaction mixture gave crude $(Acr)_{0.77}PEG_{1900}$ as an opalescent, colourless, thick oil.

Synthesis of Partially Acryloylated $(NH_2)_2PPG_{300}$(5), $(Acr)_NPPG_{300}$

$(Acr)_{0.25}PPG_{300}$ and $(Acr)_{1.10}PPG_{300}$ were prepared to be used directly in the 250 ml polymerization apparatus. Two solutions of acryloyl chloride (180 µl, 0.25 equiv.; 800 µl, 1.10 equiv.) in CH_2Cl_2 (respectively 2.5 and 11.5 ml) were each added dropwise to aliquots of $(NH_2)_2PPG_{300}$ (3.70 ml, 9.0 mmol) stirred at 0°C. When the additions were complete, the two reaction mixtures were stirred for 1 h at 20°C and concentrated to give crude $(Acr)_{0.25}PPG_{300}$ and $(Acr)_{1.10}PPG_{300}$ as colourless oils.

$(Acr)_{0.77}PPG_{300}$ was prepared to be used directly in the 850 ml polymerization apparatus. A solution of acryloyl chloride (1.9 ml, 0.77 equiv.) in CH_2Cl_2 (25 ml) was added dropwise to a mixture of $(NH_2)_2PPG_{300}$ (12.5 ml, 30.3 mmol) and CH_2Cl_2 (2.5 ml) stirred at 0°C. When the addition was complete, the reaction mixture was stirred for 1 h at 20°C and concentrated to give crude $(Acr)_{0.77}PPG_{300}$ as a colourless oil.

$(Acr)_{0.50}PPG_{300}$ was prepared to be used portionwise in the 850 ml polymerization apparatus. A solution of acryloyl chloride (5 ml, 0.50 equiv.) in CH_2Cl_2 (70 ml) was added dropwise to a mixture of $(NH_2)_2PPG_{300}$ (50 ml, mmol) and CH_2Cl_2 (70 ml) stirred at 0°C. When the addition was complete the reaction mixture was stirred for 1 h at 20°C and concentrated to give crude $(Acr)_{0.50}PPG_{300}$ as a colourless oil.

Synthesis of Partially Acryloylated $(NH_2)_2PPG_{130}$(6), $(Acr)_{0.77}PPG_{130}$

$(NH_2)_2PPG_{130}$ (11.55 ml, 47.7 mmol) was acryloylated with acryloyl chloride (3 ml, 0.77 equiv.) under the same conditions as those described for the preparation of $(Acr)_{0.77}PPG_{300}$. After drying, it was used directly in the 850 ml polymerization apparatus.

Typical Polymerization Procedure Using 850 or 250 ml Laboratory-Scale Suspension Polymerization Apparatus

A mixture of *n*-heptane–carbon tetrachloride (6:4, v/v, 470 or 138 ml) was purged with argon for 5 min in the polymerization flask. The solution was warmed to 70 °C and the stirring speed adjusted to 650 r.p.m.

During this period a mixture of the Acr_NPEG- and Acr_NPPG- monomers (see Table 1) in water (95 or 28 ml) was purged with argon. *N,N*-Dimethyl acrylamide (DMA) or acrylamide was added and after a further 5 min purging, solutions of sorbitan monolaurate (300 or 180 mg) in DMF (2.5 ml or 730 µl) and ammonium persulphate (705 or 200 mg) in water (2.5 ml or 730 µl) were added to the mixture of monomers which was then rapidly poured into the polymerization flask. After 2 min, tetramethyl ethylenediamine (TEMED) (2 ml or 600 µl) was added to the mixture and the sticky point was reached within 30 s. After about 5 min, some resin which occasionally accumulated in the top of the polymerization flask during the sticky period was re-suspended in the reaction mixture by stirring at 1500 r.p.m. (20 s) and the reaction was allowed to proceed under 650 r.p.m. stirring at 70–75°C for 2–3 h. The cooled resin was filtered off and washed twice with two volumes of ethanol then with water and passed through a steel net (1 mm^2 holes). It was transferred back to the filter, washed twice with two volumes of ethanol and dried successively under low vacuum (water pump) and high vacuum (freeze-dryer) for a period of two days.

Bromophenol Blue Assay of the $NH_2 \cdot HCl$ Loading

Molar Bromophenol Blue Absorbance ($\varepsilon_{602.5}$) 1,7-diaza-(5,4,0)-bicycloundec-1,8-ene (DBU) in DMF (0.1 M, 20 µl) was added to 50 µl of a solution R of bromophenol blue acid (0.010 M) in distilled DMF and the volume was brought to 5 ml with DMF (solution R'). Aliquots, 50–300 µl of solution R' were diluted to 2 ml with DMF and a reference curve of the absorbance at 602.5 nm ($A_{602.5}$) vs. the concentration of bromophenol blue (c_n) was determined. This was reproduced 23 times and for each reference curve obtained (n = 1–23) the molar absorbance (ε_n) and its standard deviation (s_n), obtained from the Beer–Lambert–Bouguet law, Eq. (1), were calculated by linear regression:

$$\varepsilon_n = \frac{A_{602.5}}{bc_n} \tag{1}$$

where b is the cell thickness (cm), and c_n is measured in mol l^{-1}. Under these conditions the bromophenol blue molar absorbance $\varepsilon_{602.5}$ was given by Eq. (2):

$$\varepsilon_{602.5} = \frac{\sum_{n=1}^{n} \varepsilon_n}{n} \pm \frac{1}{n}\sqrt{\sum_{n=1}^{n}(s_n)^2} = (1080 \pm 5) \times 10^2 \, l \, mol^{-1} \, cm^{-1} \tag{2}$$

where n is the number of reference curve, ε_n is the molar absorbance calculated for reference curve n (1 mol^{-1} cm^{-1}) and s_n is the molar absorbance standard deviation calculated for the reference curve n (1 mol^{-1} cm^{-1}).

Sample Preparation. A sample of the resin was washed successively with a solution of DBU in DMF (2%), DMF and dichloromethane, and dried overnight on the freeze-dryer.

Table 1 Monomer Composition of Prepared Resins

Resin composition (g)	Resin Type								
	A				B				C
	7	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
$(Acr)_2PEG_{1900}$	7.35	25	25	7.35	–	–	–	–	25
$(Acr)_NPEG_{1900}$	–	–	–	–	17.2	17.4	60	17.8	–
$(Acr)_NPPG_{300}$	3.81	13.8	14.2	4.48	–	–	–	–	–
$(Acr)_NPPG_{130}$	–	–	–	–	–	–	–	–	14.3
N[a]	0.25	0.50	0.77	1.10	0.25	0.50	0.77	1.10	0.77
DMA	1.39	4.81	4.81	1.39	–	–	–	–	4.81
Acrylamide	–	–	–	–	2.94	<2.94	10	2.94	–
Scale (ml)	250	850	850	250	250	250	850	250	850

[a] *N*: molar equivalent of acryloyl chloride used for the preparation of the partially acryloylated monomers $(Acr)_NPPG_{300}$, $(Acr)_NPEG_{1900}$ or $(Acr)_NPPG_{130}$ polymerized in resins **7–10**, **11–14** and **15**

Assay. DBU in DMF (0.1 M, 20 µl) was added to a freshly prepared solution B of bromophenol blue acid (50 µl, ≈ 0.010 M) in distilled DMF and the mixture was diluted to 5 ml with DMF (solution B'). DMF (1.9 ml) was added to 100 µl of the solution B' and the absorbance at 602.5 nm $A^i_{602.5}$ was measured.

Solution B (1 ml) was added to a weighed sample of the resin (10–20 mg) and the mixture was agitated for 45 min at room temperature. A solution of DBU in DMF (0.1 M, 20 µl) was added to the supernatant S (50 µl) and the mixture was diluted to 5 ml with DMF (solution S'). DMF (1.9) ml was added to 100 µl of the solution S' and the absorbance at 602.5 nm $A^f_{602.5}$ was measured.

The loading [NH_2·HCl] of the resin was then obtained from Eq. (3):

$$[NH_2 \cdot HCl] = \frac{2(A^i_{602.5} - A^f_{602.5})}{m\varepsilon_{602.5}} \qquad (3)$$

where $A^i_{602.5}$ and $A^f_{602.5}$ are the measured absorbance, $\varepsilon_{602.5}$ is the molar absorbance of bromophenol blue (1 mol^{-1} cm^{-1}) and m is the resin sample weight (g).

The determined loadings are presented in Table 2.

Swelling Tests. A weighed amount of resin was placed in a syringe equipped with a sintered Teflon filter and swelled in the appropriate solvent. As schematically represented in Fig. 1, a solvent flow was generated by applying a constant suction at the syringe outlet. The suction was regulated to obtain various flow rates for which the bed volumes ($V_0 - V_2$) were measured. The excess solvent was drained, the syringe was equipped with a piston and, as schematically represented on Fig. 1, a pressure of 227 N (51 p.s.i.) was applied by means of a 4.5 kg (10 pound) weight carefully placed on the piston (diameter 1.25 cm, 0.5 inch). The compressed bed volume (V_c) was measured and the piston was slowly released, allowing the resin to re-expand, so giving the minimum non-compressed bed volume (V_m). The bed volumes measured were then related to the sample weight to obtain the resin swelling abilities

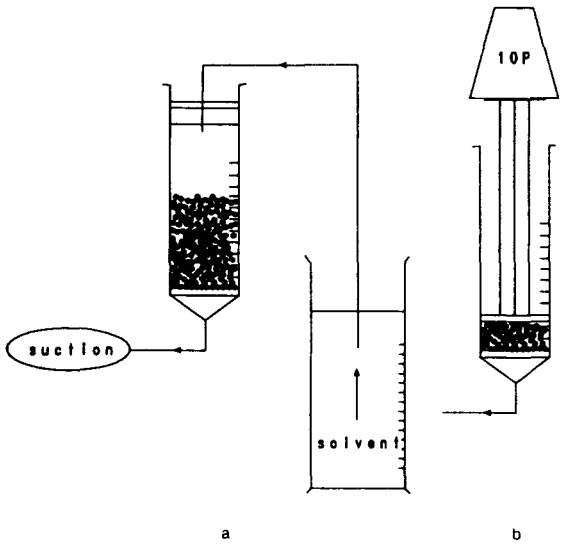

Fig. 1. Method for the evaluation of resin swelling capacity. (a) A controlled flow of solvent was established in a syringe and the bed volume was measured. (b) A controlled mechanical pressure was applied to the resin and bed volume was measured.

Table 2 Results of the Polymerization

Resin	Resin Type								
	A				B				C
	7	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
[NH_2·HCl] (mmol g^{-1})	0.11	0.19	0.23	0.24	0.15	0.155	0.17	0.17	0.36
m_{th}[a] (g)	9.56	35.3	37.8	11.94	6.14	8.86	40.7	15.67	38.1
m (g)	6.33	28.0	31.8	9.36	3.73	6.81	36.5	12.66	29.9
yield (%)	66	79	84	78	61	77	90	81	78
Molar % mono-acryloylation[b]	12	22	29	31	11	16	25	32	29
Molar % *bis*-acryloylation[b]	7	14	24	39	7	17	27	39	24
Molar cross-linking (%)	21	23	25	29	1.5	3.5	5	7	27

[a] Calculated from Eq. (8).
[b] Calculated from Eq. (9).

Table 3 Swelling Properties of Resins 7, 9, 10 and 12–15 in DMF

Swelling (ml g^{-1})	Conditions	Resin Type						
		A			B			C
		7	**9**	**10**	**12**	**13**	**14**	**15**
s_0	0 ml min^{-1}	17	12	13	18	13	13	12
s_1	1–3 ml min^{-1}	17	12	13	18	13	12	11
s_2	20–30 ml min^{-1}	15	11	12	17	12	11	10
s_c	51 p.s.i.	4	4	5	5	4	5	4
s_m	re-expanded	9	7	7	7	7	7	7
Molar cross-linking (%)		21	25	29	3.5	5	7	27

Table 4 Swelling Properties of Resins 7, 9, 10 and 12–15 in H^2O

Swelling (ml g^{-1})	Conditions	Resin Type						
		A			B			C
		7	**9**	**10**	**12**	**13**	**14**	**15**
s_0	0 ml min^{-1}	19	–[a]	–[a]	44	22	20	–[a]
s_1	1–3 ml min^{-1}	19	11	15	42	21	18	12
s_2	20–30 ml min^{-1}	–[b]	10	–[b]	–[b]	18	14	10
s_c	51 p.s.i.	5	5	4	16	10	7	5
s_m	re-expanded	9	9	10	20	14	13	9
Molar cross-linking (%)		21	25	29	3.5	5	7	27

[a] Many floating beads rendered the measurement impossible.
[b] Turbulence disturbed the resin bed at flow rates in excess of 7 ml min^{-1}.

under constant flow rates ($s_0 - s_2$), compression (s_c) or as its minimum swelling capacity (s_m). The results obtained are presented in Table 3 and Table 4 for resins **7**, **9**, **10** and **12–15** swelled in DMF or water.

Size Distribution of the Beads. Samples of resins **9** and **13** were swelled in DMF or water and placed under a macro fluorescence stereo microscope. Colour slides were taken with the mounted camera for each sample containing calibrated steel shreds (diameters: 200 and 500 μm) as internal size references. Once developed, the slides were projected on a screen (2.55 × 1.7 m) and the diameters of the beads were measured with a ruler and related to the internal reference. The diameter size distribution of the beads for resins **9** and **13** swelled in DMF or water are presented in Fig. 2.

^{13}C-NMR Spectra and Relaxation Times T_1. A sample of resin **9** carrying the peptide Y(NO_2)FQPLDEK(ABz) covalently linked to the polymer PEG chain (**16**, Fig. 4), was swelled in deuterated DMSO. The resin slurry was transferred into the nuclear magnetic resonance (NMR) tube and in order to obtain a good packing, the excess of solvent was removed by suction using along glass pipette introduced through the resin to the bottom of the tube. ^{13}C-NMR spectra and relaxation times T_1 were measured at 62.5 and 125.77 MHz respectively on Bruker AM250 and Bruker AM500 spectrometers. The experiments were recorded at 300 K and the T_1 values were determined by the inversion-recovery method using five delay times (0.005, 0.1, 0.5, 2 and 5 s) and a relaxation delay D_1 of 3 s. The results are presented in Table 5. The T_1 values were calculated using the three-parameter exponential fitting routine available in the AM500 NMR software [33–35].

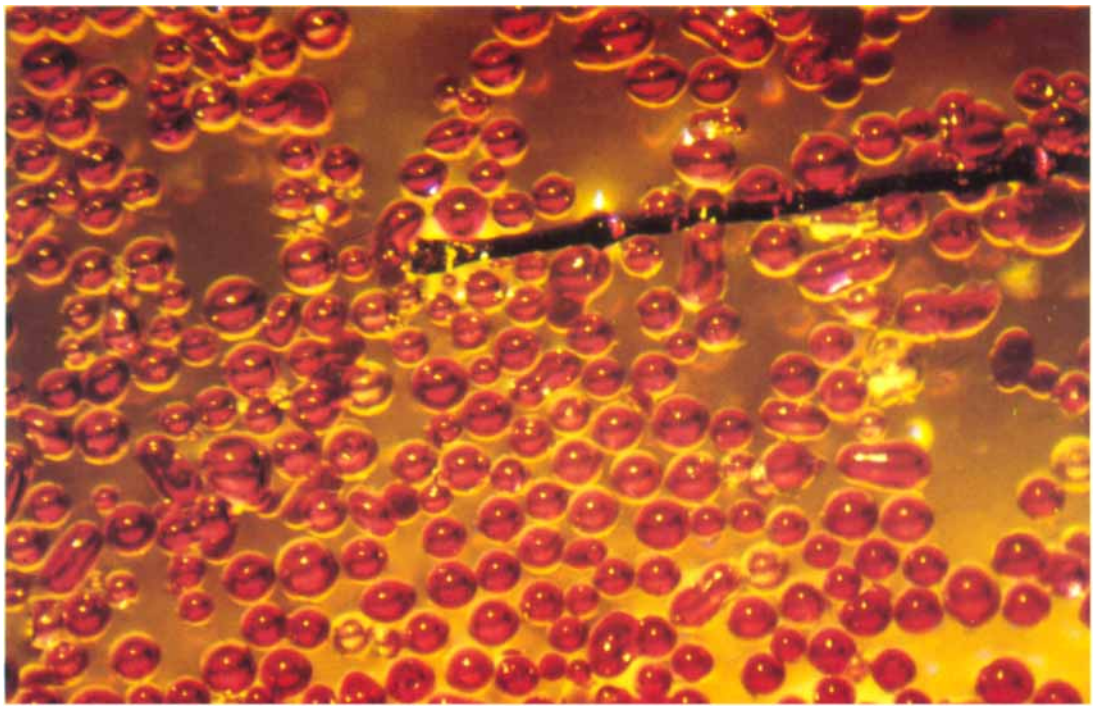

Figure 2. The size and distribution of beads were determined from slide projections of pictures of bromophenol blue stained resin containing metal threads of known diameter.

Table 5 ^{13}C-NMR Chemical Shifts and T_1 Relaxation Times for Resin Backbone and Resin-bound Peptide in Compound 16 (Figure 6)

Carbon Type[a]	δ (ppm) 125.77 MHz	T_1 (s) 62.5 MHz	T_1 (s) 125.77 MHz
PEG and PPG chains			
a	74.23	1.09	0.62
b	74.48	0.26	0.38
c	17.22	0.60	0.65
d	70.15	0.52	0.65
Polyacrylamide backbone			
e	36.40	0.60	0.64
f	34.0–36.0	0.80	0.85
g	43.5–45.0	0.46	0.41
Peptide			
C^{α}	49–60	0.19–0.36	0.17–0.23
C^{b}	27–37	0.20–0.22	0.19–0.26
CH aromatics	115–137	0.11–0.32	0.19–0.26
DBU	18–53	0.16–0.22	0.31

[a] For a_g carbon type refer to Figure 6.

Evaluation of the Pore Size for Resin 13 by Gel Permeation Chromatography

Resin **13** (9 g) was swelled in DMF, and treated twice (2 min, then 2 h) with a solution of Ac_2O (1.5 ml) in DMF (60 ml) containing *N*-ethyl morpholine (3.6 ml). During the second treatment a few agglomerated beads were separated by careful mixing. The resin was drained, washed with DMF (3 × 60 ml) and ethanol (4 × 60 ml). A sample was dried and the bromophenol blue test showed that there were no remaining free amino groups. The acylated resin was swelled in the elution buffer 50 mM Tris-HCl, pH 7.6 in 100 mM KCl, and floating particles were removed by decantation three times from 300 ml of buffer. The resin slurry was then carefully poured into a chromatographic column (diameter 1.36, height 90 cm) to give a final bed height of 85 cm, and a disc of filter paper was inserted on the top of the bed to avoid disturbing the surface while loading the samples or eluting the column. The column was eluted with a constant solvent flow rate (0.09 ml min^{-1}), and left to equilibrate for one week. The blue dextran or the protein tested was weighed (0.7–0.8 mg) and dissolved in 0.5 ml of the elution buffer. The elution profile was followed by UV-detection at 280 nm, and the volumes of elution are presented in Table 6.

Table 6 Determination of Protein Retention by Gel Permeation Chromatography on Peracetylated PEGA Resin

	MW (kD)	V_e (ml)	V_e/V_0
Blue dextrane	2000	44 (V_0)	
Bovine serum albumin	66	44	1
Ovalbumine	43	46	1.05
Carbonic anhydrase	29	47	1.07
Cytochrome C	12	52	1.18

RESULTS AND DISCUSSION

Monomers and Acryloylation

Monomer diluents such as commercially available acrylamide (**1**) or *N,N*-dimethyl acrylamide (**2**) (Fig. 3) were used in the copolymerizations in order to reduce the occurrence of neighbouring branching points in the polymer.

The cross-linker $(Acr)_2PEG_{1900}$ (**3**) was prepared from $(NH_2)_2PEG_{1900}$ by di-acryloylation with acryloyl chloride and the product was obtained pure as an amorphous powder which could be filtered off from peroxide-free diethyl ether.

The partially acryloylated monomers **4**, **5** and **6**

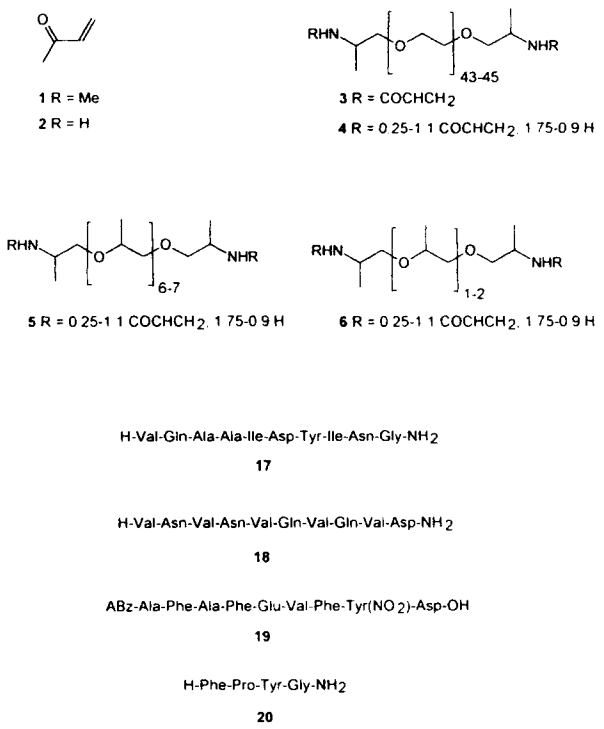

Fig. 3. The monomers used for resin preparation and the peptides synthesized in the present work.

were prepared by adding dilute dichloromethane solutions of acryloyl chloride (0.25–1.1 equiv.) to concentrated dichloromethane solutions of $(NH_2)_2PEG_{1900}$, $(NH_2)_2PPG_{300}$ and $(NH_2)_2PPG_{130}$. Because all attempts to purify the mono-acryloylated monomers failed, the crude acryloylation mixtures containing di-, mono- and non-acryloylated products were used directly in the polymerizations. The compositions of the mixtures that could not be evaluated at the monomer level were calculated after polymer synthesis and measurement of the loading $[NH_2 \cdot HCl]$ by the bromophenol blue test described below. Assuming that the acryloylation reactions were quantitative and that each acryloylated monomer was consumed to the same extent during the copolymerizations, the molar ratio of mono- and di-acryloylation could be estimated using Eqs (4)–(9) as follows:

$$n_1 + 2n_2 = N\frac{m_T}{M_T} \tag{4}$$

$$n_1 = [NH_2] \times m_{th} \tag{5}$$

$$m_{th} = n_1 M_1 + n_2 M_2 + m_3 \tag{6}$$

$$M_T = M_0 + N \times M_{AcrCl} \tag{7}$$

where M_T is the average molecular weight of partially acryloylated monomer used in the polymerization (g mol^{-1}); M_0 is the average molecular weight of non-acryloylated monomer (g mol^{-1}); M_{AcrCl} is the molecular weight of acryloyl chloride (g mol^{-1}); N is the number of molar equivalent of acryloyl chloride used in the preparation of the partially acryloylated monomer; m_T is the weight of partially acryloylated monomer used in the polymerization (g); M_1 and n_1 are the average molecular weight and number of moles of mono-acryloylated monomer contained in the partially acryloylated monomer (g mol^{-1} and mol); M_2 and n_2 are the average molecular weight and number of moles of di-acryloylated monomer contained in the partially acryloylated monomer (g mol^{-1} and mol); m_{th} is the theoretical weight of polymer expected (g); $[NH_2 \cdot HCl]$ is the loading measured by the bromophenol blue test (mol g^{-1}); m_3 is the total weight of cross-linker **3** and diluent (acrylamide or dimethyl acrylamide) used in the polymerization (g).

Combination of Eqs (4)–(6) gave Eq. (8), allowing the calculation of the theoretical weight of polymer m_{th} (Table 2):

$$m_{th} = \frac{0.5Nm_T M_2 + m_3 M_T}{M_T + M_T[NH_2 \cdot HCl](0.5M_2 - M_1)} \tag{8}$$

Inserting this result in Eq. (6) gave the number of moles of mono-acryloylated monomer n_1 which when inserted in Eq. (5) gave the number of moles of di-acryloylated monomer n_2. The molar ratio of mono- and di-acryloylated monomers contained in the partially acryloylated mixture could then be calculated for each resin (Table 2) using Eq. (9):

$$R_i = n_i \frac{M_T}{m_T} \times 100 \tag{9}$$

where R_i is the molar ratio of mono- ($i = 1$) or di-acryloylated ($i = 2$) monomers contained in the partially acryloylated resin component; n_i is the number of moles of mono- ($i = 1$) or di-acryloylated ($i = 2$) monomer.

In Fig. 4 the molar ratio of R_1 and R_2 of mono- and di-acryloylated monomers contained in partially

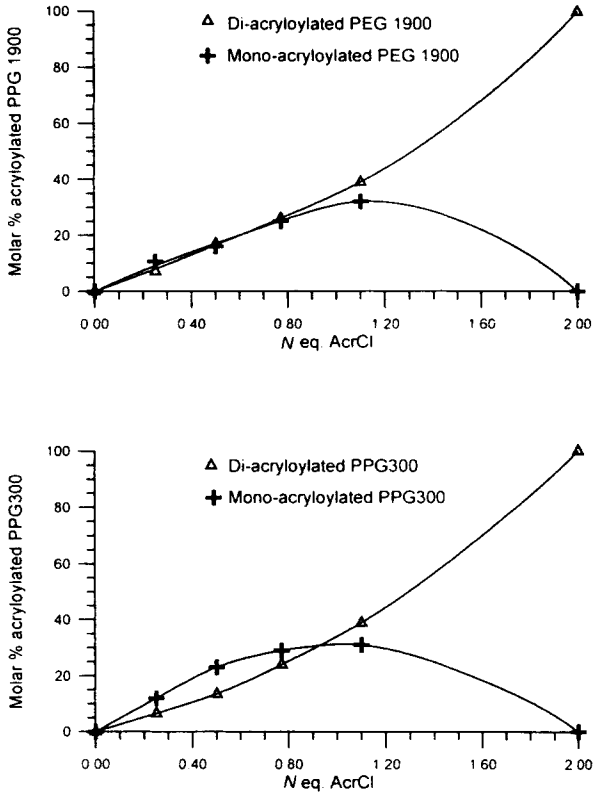

Fig. 4. The ratios between mono- and di-acryloylation (determined as described in the text) and their dependency on the amount of acryloyl chloride used.

acryloylated mixtures are presented as a function of the number of equivalent acryloyl chloride used in the partial acryloylation of $(NH_2)_2PPG_{300}$ (A) and $(NH_2)_2PEG_{1900}$ (B) samples. These curves clearly show that the amino groups of non- and mono-acryloylated $(NH_2)_2PPG_{300}$ or $(NH_2)_2PEG_{1900}$ derivatives have an equal reactivity towards acryloyl chloride and the quantitative synthesis of mono-acryloylated compounds by using only one equivalent of the acylating agent was considered impossible. It was also observed that the reaction with acryloyl chloride was not occurring under adiabatic conditions where reagent concentrations would approach homogeneity throughout the reaction mixture, leading to the preparation of 50% mono-acryloylated monomer using one equivalent of acryloyl chloride. In both partially acryloylated $(NH_2)_2PPG_{300}$ and $(NH_2)_2PEG_{1900}$ preparations the mono-acryloylation was greatest when using 0.8–1.2 equivalents of acryloyl chloride. Therefore in one series of resins in which only the amount of acylating agent is varied, the loading will be maximum when using 0.8–1.2 equivalents of acryloyl chloride. In addition, the molar cross-linking of each resin will increase with the amount of di-acryloylated monomer obtained during the partial acryloylation. Therefore, in order to minimize this cross-linking the partial acryloylation will be best performed using less than 0.8 equivalent of acryloyl chloride.

Polymerizations

Copolymerizations were performed by inverse suspension polymerization [36] using sorbitan monolaurate as a droplet stabilizer. The polymerizations were initiated by addition of bis-ammonium-peroxodisulphate and promoted with *N,N,N′,N′*-tetramethylethylene diamine. To allow suspension stabilization during stirring of the reaction mixture, the promoter was added after 2 min reaction time and the sticky point was quickly passed. As can be seen in Table 1, the resins prepared (**7–15**) can be divided in three types according to their composition:

(i) In type A (resins **7–10**), the monomer diluent dimethyl acrylamide (**1**) and the cross-linker $(Acr)_2PEG_{1900}$ (**3**) were copolymerized with partially acryloylated PPG_{300} mixtures.

(ii) In type B (resins **11–14**), crude partially acryloylated $(NH_2)_2PEG_{1900}$ mixtures containing the cross-linker $(Acr)_2PEG_{1900}$ and the functionalized monomer $(Acr)PEG_{1900}$ were copolymerized with acrylamide (**2**).

(iii) In type C, resin **15** was made in the same way than resin **9** but using 77% partially acryloylated $(NH_2)_2PPG_{130}$ instead of the longer $(NH_2)_2PPG_{300}$.

Characterization of the Resins

The *loading* $[NH_2 \cdot HCl]$ of the resin represents the number of free amino groups per gram of resin. It was determined by measuring the amount of 3′,3″,5′,5″-tetrabromophenolsulphonphthalein (bromophenol blue acid) adsorbed by a sample of resin which had been treated with a 2% solution of DBU in DMF to ensure complete deprotonation of the amino groups. The absorbance at 602.5 nm was measured and related to the residual concentration of bromophenol blue in the supernatant. The amount of bromophenol blue adsorbed by the resin sample was therefore deduced by difference with the initial concentration of the bromophenol blue solution and related to the weight of the sample. The results are presented in Table 2.

In type A and type B resins the relative molar quantities of the monomers were kept identical for each resin of one type while the degree of acryloylation was increased from 0.25 to 1.1 equiv. (cf. Table 1). As expected, the loading increased with the degree of acryloylation to reach a maximum value when about 0.8 equiv. of acryloyl chloride was used. As a result from the difference in the resin composition, the maximum loading obtained for type A resin **10** was 0.24 mmol g^{-1}, while it was only 0.17 for type B resin **14**. The loading of the PEGA resin could be increased from 0.23 mmol g^{-1} (**9**) to 0.36 mmol g^{-1} (**15**) without significantly changing the properties of the resin when the same amount (15 g) of the shorter 77% acryloylated $(NH_2)_2$PPG monomer **6** was used instead of **5** in the polymerization.

The *molar cross-linking* defined by the molar percentage of cross-linking monomers contained in a resin was calculated for each resin (Table 2). As expected, the molar cross-linking increases with the degree of acryloylation of the partially acryloylated monomer. This is particularly noticeable in resins of type B in which the crosslinking only results from the amount of di-acryloylated $(NH_2)_2PEG_{1900}$ contained in the partially acryloylated monomer $(Acr)_NPEG_{1900}$. Type B resins are therefore resins with little cross-linking (<10%) as opposed to both type A and C resins (>20%), which were obtained by adding a large amount of cross-linker **3** ($(Acr)_2PEG_{1900}$) in the polymerizations.

The *swelling* of the resins could be measured under various conditions using the simple settings presented in Fig. 1. As described above, the swelling was determined with flow, under mechanical compression and as the minimum non-compressed volume. Swelling volumes were determined in DMF (Table 3), and in water (Table 4). However, very similar swelling as observed in DMF for resins of types A and C were observed in other solvents, e.g. dichloromethane, TFA, acetonitrile and DMSO, indicating that the degree of swelling allowed by complete stretching of the cross-linking PEG chains (maximum degree of swelling) had been obtained. The results showed that the swelling capacities of the PEGA resins under flow conditions both in water and in DMF decreased significantly when the cross-linking increased from 3.5 to 5% for type B resins. This difference was also observed after compression. We also observed that above these cross-linking values, their swelling capacity became fairly independent of the degree of cross-linking. Interestingly, while type A resins have almost identical swelling capacities in water and in DMF, the type B resins have almost the double swelling capacity in water than in DMF. These results, together with the observation that in DMF both type A and type B resins have comparable swelling capacities, showed that the swelling characteristics of the PEGA resins depended both on the degree of cross-linking and on the chemical nature of the diluent (acrylamide vs. dimethyl acrylamide). Resin types A and C, both highly cross-linked resins containing dimethyl acrylamide as diluent, had very analogous swelling capacities and both swelled much less in water than type B resins characterized by a low molar content of cross-linker and use of acrylamide as a diluent. It was further demonstrated that enhancing the swelling capacity of this type of resin by decreasing the cross-linking below 3.5% did not lead to a beaded resin but rather to a gel-like polymer.

The *statistical size distribution* of the beads were determined for resins **9** and **13** swelled in DMF or water. Colour slide pictures of the samples containing calibrated steel shreds (Fig. 2) were taken with a camera mounted on a stereo microscope and projected on a large screen (2.5 × 1.7 m), permitting the measurements of the beads and internal reference diameters with a ruler. The diameter size distribution of the beads for resins **9** and **13** that had swelled in DMF or water are presented in Fig. 5. As expected from the swelling capacities reported and discussed above, the size distribution of the beads of both resins were quite similar when swelled in DMF, whereas in water, the beads of resin **13** swelled much more than those of resin **9**.

The *flexibility* of the different resin components was accessed by determination of the ^{13}C-NMR relaxation parameter T_1 which is related to the internal tumbling of the particular resin component by the following equation [37]:

$$1/T_{1(DD)} = h^2\gamma_C{}^2\gamma_H{}^2r_{CH}{}^{-6}N\tau_c \qquad (10)$$

where the number of protons N on a given carbon and the local correlation time of the polymer determine T_1.

The measured T_1 values are presented in Table 5 for compound **16** (Fig. 6), composed of resin **9** carrying the peptide Y(NO_2)FQPLDEK(ABz). These values, measured at 62.5 and 125.77 MHz, showed that the PEG and PPG chains were slightly more rigid than the polyamide backbone, probably due to the

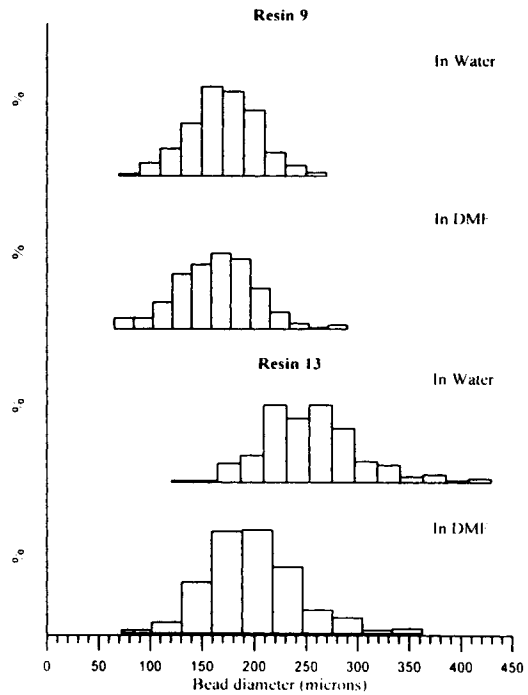

Fig. 5. The bead size distributions for PEGA-resin **9** and **13** swelled in water and DMF determined from slide projections (cf. Fig. 2).

helical preference of the PEG and PPG chains resulting from the *gauche* effect [38–40]. The T_1 values measured were not field-dependent, indicating that the internal motion corresponded to an approximate correlation time τ_c of 10^{-10} s. Nevertheless, both polyamide and polyethylene glycol or polypropylene glycol chains were shown to be more flexible than the peptide chain (longer T_1). The ^{13}C-NMR T_1 values observed for the peptide were comparable to those observed in solution for a medium-size peptide [41].

Mass transfer in PEGA resin has been shown [8] to be extremely fast, allowing reaction times of 1–2 min for uncomplicated amino acid acylations, whereas β-branched amino acids required 40–60 min for the reactions to go to completion. This property allowed the synthesis of various peptides and glycopeptides such as the acyl carrier protein (65–74) **17** [8] (Fig. 3). There was no aggregation observed, and in the extreme case of synthesis of VNVNVQVQVD (**18**) without amide protection, long reaction times secured complete acylations whereas other flow resins failed to give the right product [20]. The resin **9** was used for the multiple column peptide synthesis (MCPS) of fluorogenic protease substrates, e.g. glutamic acid specific protease substrate **19** and

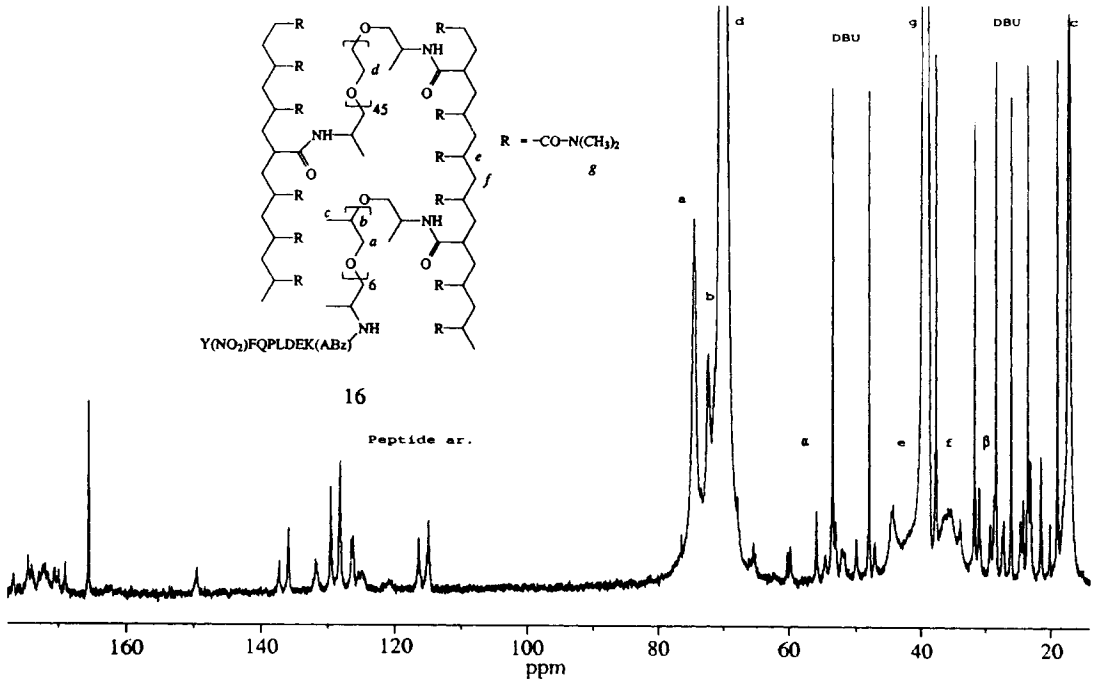

Fig. 6. Structure of the PEGA-resin with indexes referring to Table V for measured T_1 values and the corresponding ^1H-NMR spectrum.

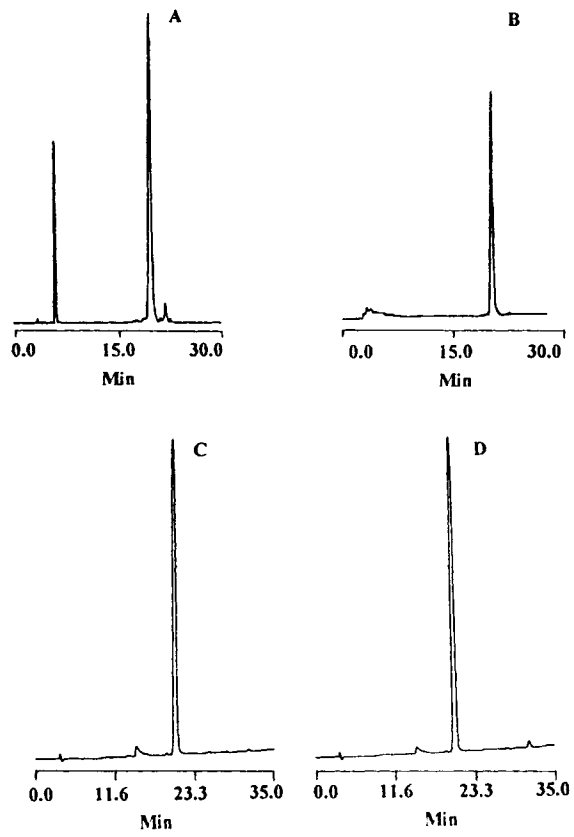

Fig. 7. Chromatograms of crude (A) and purified (B) peptide **19** synthesized by MCPS on resin **9** and of crude peptide **20** synthesized on resin **9** in 81% yield (C) and **15** in 89% yield (D) respectively.

the chromatograms of the crude and purified material are shown in Fig. 7. The peak at 19.6 min gave the correct sequence analysis, amino acid analysis and mass-spectrum by electron spray mass spectrometry (ESMS). The yield of crude product was quantitative and after preparative reversed-phase high-performance liquid chromatography (HPLC) purification, 48% of pure **19** was isolated. Similarly 81% and 89% yields were obtained when peptide **20** was synthesized on resins **9** and **15**, respectively. Chromatograms of the crude products are shown in Fig. 7. Resins **9** and **13** were found to be excellent for the synthesis of libraries in the MCPS-library generator [42] to afford libraries of internally quenched fluorogenic peptides [21]. The mass transfer of bigger molecules such as protein and enzymes in resin **13** was evaluated by gel permeation chromatography. A sample of resin **13** was acylated

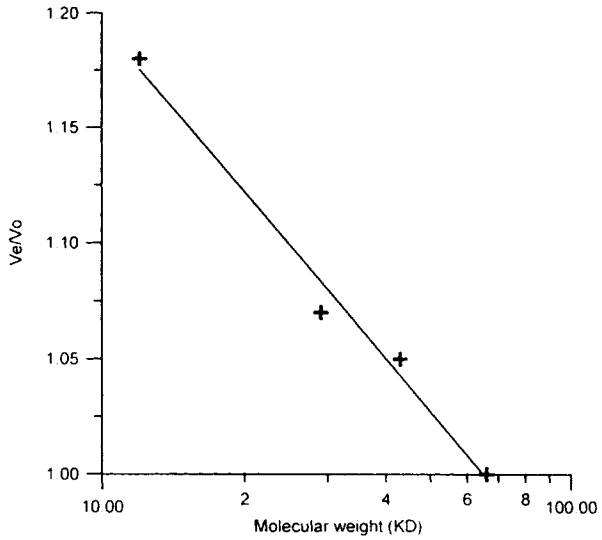

Fig. 8. Semi-logarithmic plot of elution volume ratios, V_e/V_O, against protein molecular weights. Exclusion occurs at approximately 65 kD.

to avoid ionic interactions with the proteins, swelled in the elution buffer (Tris-HCl, pH 7.6) and placed into a chromatographic column. After equilibration, blue dextran or protein was dissolved in the elution buffer and the elution profile was followed by UV-detection at 280 nm. The column void volume was measured with blue dextran (2000 kD) and the volumes of elution for globular proteins of various sizes were measured. The results presented in Table 6 showed that while bovine serum albumin (66 kD) was excluded from the polymer, smaller proteins were allowed to penetrate the network.

When the ratio V_e/V_0 was plotted as a function of log(molecular weight) we obtained a straight line (Fig. 8) as should be expected in gel chromatography, showing that the PEGA resin behaved well for gel permeation chromatography.

Enzyme Reactions

A known substrate for subtilisin A, Y(NO_{2A})-FQPLDEK(ABz) was synthesized on resin **9** and the ability of the enzyme, which has a molecular weight of 27 kD, to enter into the resin and cleave the substrate was assayed by sequence analysis. The resin was reacted with a 1 μM solution of subtilisin A. After 1 h the peptide was more than 80% cleaved according to sequence analysis and the reaction was quantitative after 24 h. The cleavage was found to be

quantitative and the enzyme must therefore have been allowed to enter all parts of the resin beads. Portion mixing libraries [18, 43] of fluorogenic peptide substrates were synthesized and used for the determination of the substrate specificities of proteases as described elsewhere [21].

Similar results with complete permeability towards enzyme were obtained for a reaction of β-1-4-galactosyl transferase (48–51 kD) and a resin (**9**) bound substrate as will be described in a forthcoming publication [44].

CONCLUSION

A new type of peptide synthesis resin with interesting properties for organic synthesis and biochemical use has been prepared. The resin is formed by easy radical polymerization of acryloylated derivatives of PEG. The high swelling potential of the PEGA resin yields tense beads that are completely flow-stable, yet have a very flexible polymer network, allowing a very high rate of mass transfer. Inclusion of components other than the acrylamides or different length of cross-linker may yield materials which can be used for many other purposes, e.g. chromatography or affinity columns.

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