A Physically Supported Gel Polymer for Low Pressure, Continuous Flow Solid Phase Reactions. Application to Solid Phase Peptide Synthesis

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Summary Copolymerisation of dimethylacrylamide, ethylene bisacrylamide, and acryloylsarcosine methyl ester within the pores of rigid macroporous inorganic particles provides supports suitable for continuous flow solid phase peptide synthesis and for other applications.

SOLID phase peptide synthesis[1] is conventionally a discontinuous process in which insoluble gel-supported peptide is treated sequentially with acylating and deprotecting agents in a shaken or stirred reaction vessel. The excess of reagents is removed by filtration and repeated washing steps. As has been recognised by others (*e.g.* refs. 2,3) substantial advantages might accrue if this and similar processes could be carried out in flow systems. Removal of the excess of reagents would be quicker, more efficient, and more economical, control of the process would be much simplified, and greater opportunities would be presented for continuous monitoring of the several chemical reactions with the possibility of true automated feedback control. Attempts several years ago to develop such a system in this laboratory using our normal polyamide gel supports[4] were unpromising. Packing down of the resin occurred under pumped flow conditions with destruction of its open matrix character and the generation of excessively high pressures. Similar high pressures have been encountered elsewhere with polystyrene gels. Use of steel reaction columns and h.p.l.c.-type pumping systems have been described,[2,3] and pressures up to 10,000 psi mentioned.[3] We now describe a new type of composite gel support which appears to provide a general solution to these problems.

Our new support consists of cross-linked and functionalised polydimethylacrylamide gel held within the pores of inert, rigid, macroporous particles. Inorganic particles have proved to be particularly suitable and the work described here utilises fabricated kieselguhr[5] as the skeleton into which the non-rigid, functional gel is formed. The pores of the support are very large (several thousand Angströms diameter), and so the porosity of the composite is essentially that of the organic gel while the mechanical properties (*e.g.* size, rigidity, strength), are those of the inorganic matrix. Other rigid carriers, *e.g.* macroporous polystyrene and other organic polymers may also be used,† and other gels, *e.g.* the conventional styrene–divinylbenzene copolymer, have also been polymerised within the kieselguhr matrix.

Typically, a solution of the acrylate monomers was polymerised with the beads under anaerobic conditions. Thus an aqueous solution of ammonium persulphate (10% w/v, 7·5 ml) was added to a solution of freshly distilled dimethylacrylamide (10 g), ethylene bisacrylamide (1·17 g) and freshly distilled acryloylsarcosine methyl ester (0·85 g) in dimethylformamide (DMF) (16 ml) and water (25 ml). The mixture was soaked into fabricated kieselguhr particles[5] (29 g; 355—500 μ) and the polymerisation allowed to proceed for 2½ h at room temperature. The resulting composite was washed with water and agglomerated particles separated and unbound polymer removed. The beads were then washed in acetone–water (1 : 2), acetone, and diethyl ether and dried *in vacuo*. The product (30·9 g) was functionalised with carboxymethyl groups to the extent of 0·046 mequiv./g (sarcosine content). By the appropriate choice of conditions, composites with a sarcosine content of up to 0·237 mequiv./g have been prepared.

Columns of the polydimethylacrylamide support generate negligible back pressure at flow rates (DMF) of 100 ml h^{-1} cm^{-2}. Presumably most of the liquid flow occurs around the particles and reactant penetration of the internal gel matrix is largely diffusion controlled. Peptides thus far assembled using the new support include the customary Merrifield–Dorman test tetrapeptide, Leu.Ala.Gly.Val, its isomer, Leu.Ala.Val.Gly, an undecapeptide amide from the HLA–DR antigen sequence,[6] and several peptide amides from the gastrin series. Its use is illustrated here by the synthesis of the gastrin-related octapeptide amide, Glu.Ala.Tyr.Gly.Trp.Leu.Asp.Phe.NH_2 (**1**). The procedure employed is essentially that of our established fluorenyl-methoxycarbonyl–polyamide method[7] adapted to the flow system.

The foregoing polydimethylacrylamide–kieselguhr composite support (3·45 g, 8 ml) was functionalised by reaction with ethylene diamine (15 ml) for 16 h, the resin filtered and washed well with DMF, and part (6·5 ml, *ca.* 0·12 mequiv.) of the slurry packed in a 15 mm diameter glass column. The column was connected to a simple polytetrafluoroethylene valving and pumping system permitting reagent selection, flow, and recirculation. Effluent from the column was monitored continuously at 308 or 312 nm. Freshly prepared[7] fluorenylmethoxycarbonyl (Fmoc)-norleucine anhydride (internal reference amino-acid) (0·5 mmol)‡ in DMF was added to the top of the column and recirculated at a flow rate of 3·3 ml min^{-1} for 1 h. A resin sample withdrawn after 30 min gave a negative ninhydrin test. The column was washed with DMF (15 min), Fmoc groups cleaved with 20% piperidine–DMF (10 min), and the resin washed again

† This aspect is under investigation by Dr. D. C. Sherrington and his colleagues at the University of Strathclyde, Scotland.

‡ In more recent experiments using a higher loading resin, efficient amino-acid incorporation has been obtained using only a *ca.* 2-fold excess of activated derivatives (E. Brown and R. C. Sheppard, unpublished).

with DMF (30 min). Further acylation steps were then carried out with 0·5 mmol of the trichlorophenyl ester of *p*-hydroxymethylbenzoic acid (peptide resin linkage agent) in the presence of an equivalent amount of hydroxybenzotriazole, and then Fmoc-phenylalanine anhydride (0·5 mmol) in the presence of *N*-methylmorpholine (1 equiv.) and *p*-dimethylaminopyridine (0·1 equiv.)[8] (esterification of the first amino-acid residue to the resin). The remaining seven residues of the octapeptide sequence (**1**) were added successively using Fmoc-amino-acid anhydrides with t-butyl side chain protection as appropriate. After addition of the glycine residue, subsequent steps were program-controlled with acylation time 51·2 min (ninhydrin test at 25·6 min), DMF wash 12·8 min, deprotection 9·6 min, DMF wash 25·6 min, total cycle time 99·2 min. Wash times were set arbitrarily and may be considerably shortened. A typical u.v. record of one cycle of amino-acid addition and deprotection is shown in Figure 1. All ninhydrin reactions were negative at the first test.

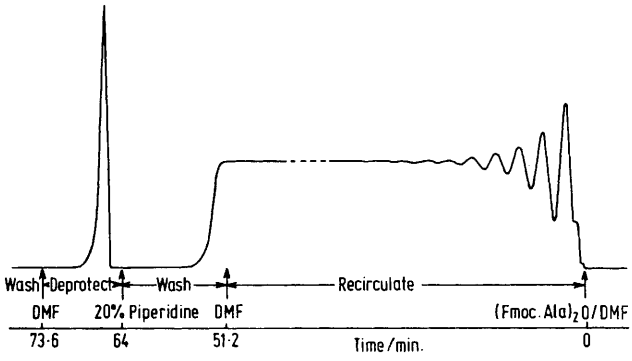

FIGURE 1. Photometric record of the column effluent during one cycle of amino-acid addition and deprotection. Flow rate 3·3 ml min^{-1}; λ = 312 nm; path length = 0·1 cm.

Samples for amino-acid analysis§ were removed after step 4 (Found: Leu, 0·98; Asp, 1·02; Phe, 1·00; Nle, 1·06), and step 8 (Found: Glu, 1·02; Ala, 0·95; Tyr, 1·01; Gly, 1·03; Leu, 0·97; Asp, 1·00; Phe, 1·00; Nle, 1·08). Side chain protecting groups were removed from the resin-bound octapeptide by treatment with 90% trifluoroacetic acid (30 min), and the amide detached with saturated methanolic ammonia (18 h). The total product (Found: Glu, 1·07; Ala, 1·02; Tyr, 1·01; Gly, 1·04; Leu, 0·98; Asp, 1·04; Phe, 1·00) (h.p.l.c., Figure 2a) was chromatographed on diethylaminoethyl-cellulose (Figure 2). The octapeptide amide recovered from the main peak (Found: Glu, 1·07; Ala, 1·02; Tyr, 1·00; Gly, 1·03; Leu, 0·99; Asp, 1·01; Phe, 1·00) was identical on h.p.l.c. (Figure 2b) and t.l.c. (BuOH–H_2O–AcOH–pyridine, 30 : 12 : 3 : 10) with the same derivative prepared during contemporary solid phase fragment condensation studies.[9] The overall yield was 47%.

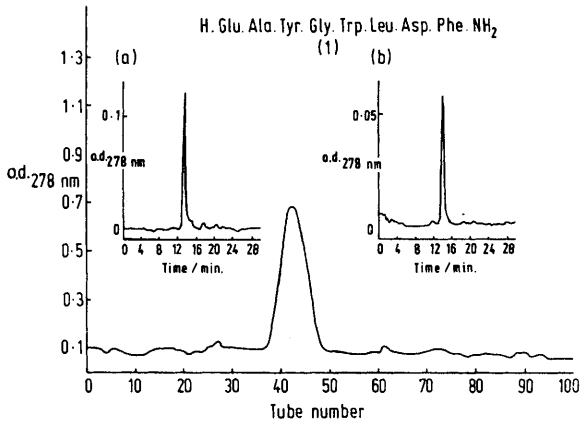

FIGURE 2. Anion-exchange chromatography of crude octapeptide amide on diethylaminoethyl-cellulose DE52 using a linear gradient of 0·01—0·5 M aqu. ammonium hydrogen carbonate, pH 8·1. Flow rate 1 ml/min; fraction size 6 ml. Insets: H.p.l.c. on μ-Bondapak C_{18} (a) before, and (b) after anion-exchange chromatography.

The novel, physically supported resin provides the basis for a simple and efficient procedure for solid phase peptide synthesis. A similar flow system has been devised for solid phase oligodeoxyribonucleotide synthesis,[10] and other applications, *e.g.* solid phase protein sequencing (*cf.* ref. 11) may be envisaged. The uniformly low pressures generated provide many advantages. No detectable compression of the gel resin occurs which might modify its open matrix character and hinder reactant accessibility. Reagent penetration and removal is rapid as shown, for example, in the spectrophotometric record of the deprotection (Fmoc group cleavage) step (Figure 1). Acquisition of analytical data during the synthesis both by resin removal and effluent monitoring is particularly easy. Computer intepretation of the spectrometric record should enable further progress towards full automation.

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§ Tryptophan was not determined.

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