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Design and Application of a New Rigid Support for High Efficiency Continuous-flow Peptide Synthesis

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A new high capacity composite support consisting of an extremely porous, rigid matrix which provides a scaffold into which a soft polyacrylamide-based gel is chemically bound has been developed and its application in low-pressure continuous-flow solid-phase peptide synthesis is described.

As an alternative to the well established, discontinuous, batch procedures[1] a very important recent development in solid-phase peptide synthesis has been the introduction of low-pressure, continuous-flow methods.[2] These allow the use of glass reaction columns and provide the advantage of on-line monitoring of repetitive reactions. Basic to any potential low-pressure flow system is the need for an appropriate support matrix. Existing gels for solid phase peptide synthesis have been found to undergo dramatic changes in bed volume during peptide assembly and to be compressible under flow conditions yielding unacceptable back pressure for use in simple packed-bed glass columns.[3] The polyamide/kieselguhr resin developed at the M.R.C., Cambridge, by Sheppard and co-workers,[4] was the first example of a support specifically designed to be utilized in a low-pressure continuous-flow mode. This composite was formed by polymerizing a polyacrylamide gel within the pores of a fabricated kieselguhr matrix. In combination with the base labile fluorenylmethoxycarbonyl (Fmoc) group[5] for N^{α} protection of the incoming amino acid, real-time u.v. spectrometric monitoring of individual acylation and deprotection reactions has been achieved. In addition, a single solvent (dimethylformamide, DMF) can be used throughout. However, two major deficiencies exist which are support related, and which are likely to inhibit its wider use, particularly in larger scale operations. Firstly, the support has low levels of substitution, typically 0.1—0.2 $mmol^{-1}$ g^{-1}, which provides relatively poor yields of peptide per column volume. Secondly, the physical stability can be rather poor, leading to the generation of fines from the fragile, fabricated structure, and also to escape of the physically trapped gel from the pores during solvation and flow.

We describe a new composite support[6] which overcomes the problems outlined above and which provides for the first time a high-capacity rigid matrix with excellent flow and physical characteristics, offering a real prospect for large-scale continuous-flow, low-pressure column operation. The composite is derived from a new class of porous polymers, developed at Unilever Research, Port Sunlight. These species, which we call Polyhipe™[7] structures, are poly(styrene-co-divinylbenzenes) formed by polymerization of a high internal phase emulsion and in this case provide 90% pore volume within the structure.

The cell size of this matrix is designed to be 20—30 μm and each cell is interconnected to give a completely open network (Figure 1). This structure, in the form of granules, is utilized as the primary scaffold into which conventional solid phase gels are chemically bound. Chemical immobilization is ensured by functionalization of the scaffold to provide double bonds at the surface of each cell. These take part in the polymerization process during which the secondary gel matrix is formed. The low density of the primary scaffold has allowed composites with an activity >1 mmol g^{-1} to be prepared by incorporation of appropriate solid-phase gels.[8] In addition to much improved synthetic capacity, the flow properties of the new

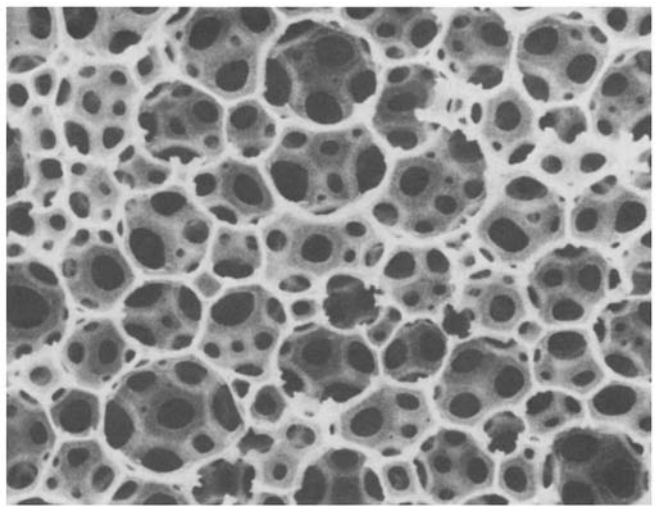

Figure 1. Polyhipes™ microarchitecture as displayed by a scanning electron micrograph (magnification ×1000).

supports are excellent and the chemical bonding of the gel produces a robust composite which generates few fines during use and ensures no loss of gel into solution. An example of the preparation of a composite containing an acryloylsarcosine methyl ester–dimethylacrylamide cross-linked gel[9] at 1 mmol g^{-1} synthetic capacity is given here. Its application for peptide synthesis is exemplified by the synthesis of leucine enkephalin and the notoriously difficult decapeptide segment from acylcarrier protein, ACP (65—74).[10]

Typically, the scaffold polymer in granular form was derivatized with aminomethyl groups *via* imidomethylation, followed by nucleophilic cleavage to form amino groups by a literature method.[11] Introduction of acryloyl groups was achieved by further reaction of the aminomethylated Polyhipe™ (2.0 g) with acryloyl chloride (10 cm^3; 0.12 mol) in a solvent mixture of tetrahydrofuran–H_2O (1 : 1; 5.0 cm^3). The reaction was carried out at 0 °C and the pH adjusted and maintained at 11 by addition of 2 mol dm^{-3} sodium hydroxide. After 4 h reaction, a ninhydrin test on the polymer indicated absence of primary amine groups. The resin was washed with methanol/H_2O followd by methanol. The formation and chemical binding of the secondary acrylamide gel inside the scaffold can be summarized as follows. Derivatized porous polymer (1 g; 425—850 μm particle size), acryloyl sarcosine methyl ester (2 g), *N*,*N*-dimethylacrylamide (5 g), ethylenebisacrylamide (0.5 g), and azoisobutyronitrile (0.1 g) were suspended in DMF (15 cm^3) in a round-bottomed flask. The mixture was purged with nitrogen for 30 min and the flask transferred to a rotary evaporator, adapted for reflux, then heated, at 60 °C for 2 h with sufficient agitation to maintain discrete particles. The composite product (6.5 g) was washed exhaustively with DMF, ethanol, and ether. The resulting granules were milled and sieved to provide particles in the range 250—500 μm.

For peptide synthesis the support was further functionalized by reaction with ethylenediamine (20 cm^3 g^{-1}) for 16 h, followed by exhaustive washing with DMF and ether. The composite (2 g) was transferred to two separate 1 × 10 cm glass columns. Each column was slurry-packed with support (1 g; 1 mmole substitution level) in DMF and no column back pressure was observed under standard flow conditions, utilizing a commercial continuous-flow peptide synthesizer (Milligen 9020, semi-automatic instrument).

For each column Fmoc-norleucine-pentafluorophenyl ester[12] (Fmoc-Nle-OPfp) (3 mmol) was injected onto the column as a DMF solution and recirculated at a flow rate of 3 cm^3 min^{-1} for 1 h. A resin sample withdrawn after 30 min gave a negative ninhydrin test. Following a standard wash cycle with DMF (15 min), the Fmoc group was cleaved with a solution of 20% piperidine in DMF (7 min) and the column washed again with DMF (15 min). Further acylation steps were then carried out with the pentafluorophenyl ester (–OPfp) of hydroxymethylphenoxyacetic acid (peptide resin linkage agent) (3 mmol) in the presence of an equivalent molar quantity of hydroxybenzotriazole (HOBt) and in case of leucine enkephalin, Fmoc-Leu-anhydride (3 mmol) in the presence of 4-dimethylaminopyridine (0.1 mmol) was introduced onto the column to provide esterification of the first amino acid to the support. For ACP (65—74) Fmoc-glycine was attached in an identical manner. Samples removed for amino acid analysis indicated 0.90 and 0.91 mmol g^{-1} esterification yield respectively. For leucine enkephalin, the remaining four amino acids, Fmoc-Phe, Fmoc-Gly, Fmoc-Gly and Fmoc-Tyr-(Bu^t), were added successively as –OPfP esters (3 mmol) with HOBt assistance. All acylations were complete in 30 min as indicated by the ninhydrin test and all deprotection peaks, derived from the effluent stream monitored at 314 nm, were of equal integration area. For ACP (65—74), the remaining nine amino acids, Fmoc-Asn, Fmoc-Ile, Fmoc-Tyr-(Bu^t), Fmoc-Asp-(OBu^t), Fmoc-Ile, Fmoc-Ala, Fmoc-Gln and Fmoc-Val were added using the same protocol, *i.e.* OPfp esters (3 mmol) in the presence of HOBt. Again, acylations were judged to be complete in 30 min and deprotection peaks observed to be consistent.

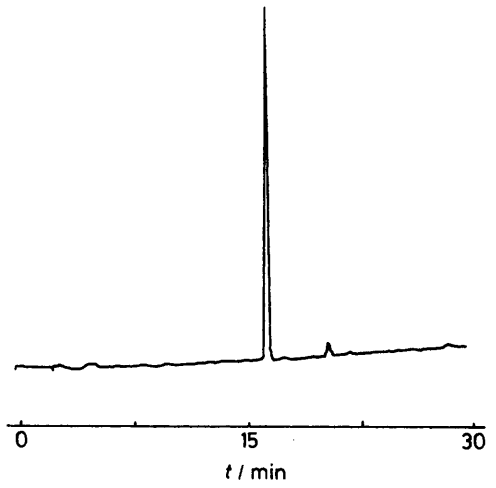

Figure 2. Analytical h.p.l.c. of crude leucine enkephalin on reverse phase (Spherisorb ODS). Solvent A = 0.1% TFA in H_2O, solvent B = 0.1% TFA in MeCN; linear gradient from 10% B in A to 60% B in A over 30 min; flow rate 1.5 cm^3/min; u.v. monitor at 220 mn.

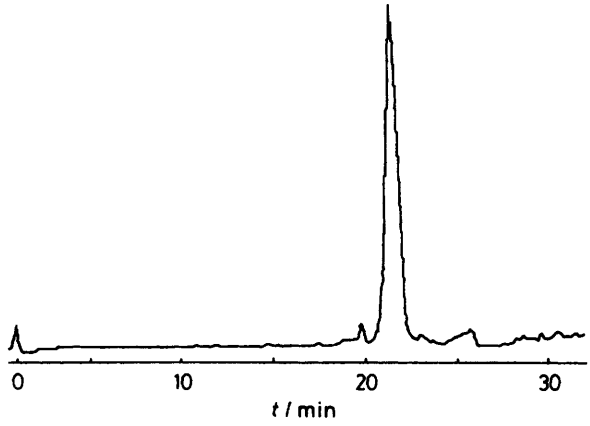

Figure 3. Analytical h.p.l.c. of crude ACP (65—74) on reverse phase (Waters-Novapak C-18); conditions as in Figure 2.

Detachment of both peptides was carried out by suspending the peptide-resin in trifluoroacetic acid (TFA) –H_2O (95 : 5; 20 cm^3) for 2 h with occasional magnetic stirring. Following filtration the solvent was removed under reduced pressure and in both cases the residue triturated with ether. The yields for leucine enkephalin and ACP (65—74) were 0.48 and 0.91 g respectively. Amino acid analyses were as follows: Leu-enkephalin (Found: Leu 1.00; Phe 0.97; Gly 2.05; Tyr 0.93); ACP (65—74) (Found: Gly 1.00; Asp 1.93; Ile 1.95; Glu 1.03; Ala 1.98; Val 0.91; Tyr 1.02). H.p.l.c. of both peptides (Figures 2 and 3) confirmed a major peak in each case which corresponded to the desired peptide.

The new composite has been shown to have a number of distinct advantages over existing supports when applied in continuous-flow synthesis. The structure provides for excel-

lent flow properties and reagent/solvent accessibility under low pressure. The chemical binding of the secondary matrix ensures no loss of gel even under extreme solvent flow conditions and the high porosity of the primary scaffold has allowed high peptide loadings to be attained with appropriate secondary solid-phase gels. In this case the performance of a support with 1 mmol g^{-1} capacity is described, providing greater quantities on a per gram resin basis than those previously achieved with a commercially available flow instrument and the recommended columns (1 × 10 cm). In further work, capacities up to 5 mmol g^{-1} have been found to be practical.

The performance of the composite under batch conditions is also being evaluated. In this case the mechanical strength of the supported gel provides stability to all common agitation techniques, and in addition the structure ensures excellent filtration properties.

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