A Solubilisable Polymer Support Suitable for Solid Phase Peptide Synthesis and for Injection into Experimental Animals

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New polymer supports containing cleavable cross-links are described; these supports are suitable for solid phase synthesis and can then be solubilised for further chemical or biological manipulation (including immunisation) in solution.

Most current solid phase peptide synthesis is directed towards raising antipeptide antisera in experimental animals. In favourable circumstances, these antisera cross-react with proteins containing the same linear amino acid sequence,[1] and are correspondingly important tools in modern biology. To increase the immunogenicity of short peptides, it is generally necessary to link them to large carrier molecules, usually proteins, before injection.[1] There is thus a need for polymer supports suitable for solid phase peptide synthesis which can also be used directly as carrier molecules for immunisation. We report here the design and synthesis of novel insoluble gel polymers which can be used in peptide synthesis, solubilised, and the resulting linear polymer-peptides used in further chemical or biological experimentation in solution.

CH_2=CH–$CONMe_2$ (**1**) CH_2=CH–$CONMeCH_2CO_2Me$ (**2**) CH_2=CH–CONH–CH_2CH_2–NHCO–CH=CH_2 (**3**)

CH_2=CH–CONH–CH_2CH_2–O–CR_2–O–CH_2–CH_2–NHCO–CH=CH_2 (**4**) CH_2=CH–CONH–$(CH_2)_6$–OAc (**5**)

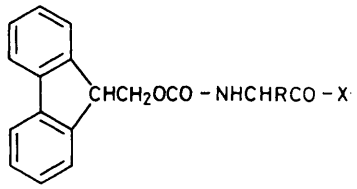
Peptide synthesis on insoluble cross-linked poly-(dimethylacrylamide) gels obtained by copolymerisation of dimethylacrylamide (**1**), acryloylsarcosine methyl ester (**2**) (functionalising agent), and ethylene bisacrylamide (**3**) (cross-linking agent), is well established.[2] Linear poly-(dimethylacrylamide) is water-soluble and we therefore constructed new poly(dimethylacrylamides) in which (**3**) was replaced by a chemically cleavable linkage. It was appropriate to use base-stable, acid-labile structures for use in combination with the fluoren-9-ylmethoxycarbonyl (Fmoc)-t-butyl peptide synthesis strategy,[3] permitting solubilisation of the polymer and simultaneous cleavage of t-butyl-based amino acid side chain protecting groups from the assembled peptide.

Early experiments used the formaldehyde-derived cross-linking agent (**4**; R = H) prepared by acryloylation of the corresponding diamine.[4] In admixture with the monomers (**1**) and (**2**), this gave a gel polymer on which the undecapeptide sequence (**6**) related to substance P could be synthesised without difficulty, but which was then only partly soluble after treatment with acidic reagents.† This is now thought to be due to additional permanent cross-linking introduced when ester groups from (**2**) were converted to aminoethyl derivatives by the usual treatment with ethylene diamine. A completely acid-soluble polymer was obtained by copolymerisation of dimethylacrylamide (**1**), the ketal crosslinking agent (**4**; R = Me), and the new functionalising agent (**5**), in a molar ratio of 17.6:1.2:1 in deoxygenated dimethylformamide solution initiated by benzoylperoxide (0.5 molar proportion). Compound (**5**) was simply prepared by successive acryloylation and acetylation of 6-aminohexanol. Polymerisation within the pores of macroporous Keiselguhr particles gave a physically supported polymer suitable for continuous flow peptide synthesis in a column reactor.[5]

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Nle-polymer
(**6**)

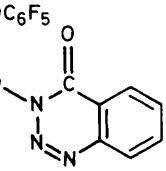
H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-Gly-polymer
(**7**)

The peptide sequences (**6**) and (**7**) related‡ respectively to the neuropeptides substance P and substance K (neurokinin A)§ were assembled on the new insoluble polymer support using Fmoc-polyamide continuous flow techniques.[5] After removal of the protecting acetyl group [from monomer (**5**)] with diaminoethane, the first amino acid residue was esterified to the resin using the Fmoc-amino acid anhydride in the presence of dimethylaminopyridine. Subsequent residues were added as Fmoc-amino acid pentafluorophenyl esters[6] (**8**)¶ in the presence of anhydrous 1-hydroxybenzotriazole catalyst [for (**7**)], or the newer 3-(3,4-dihydro-4-oxo-benzotriazinyl) esters[7] (**9**) [for (**6**)]. t-Butyl ethers, esters, or t-butoxycarbonyl derivatives were used for amino acid side



(**8**) X = OC_6F_5

(**9**) X =



chain protection as appropriate. Arginine was protected as the 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) derivative. Use of base-labile fluorenylmethoxycarbonylamino acid derivatives throughout avoids the acidic reaction conditions of conventional solid phase synthesis. In the absence of water, no evidence was obtained for significant dissolution of the polymer by weakly acidic species (hydroxybenzotriazole, pentafluorophenol *etc.*) present in the reaction mixtures. The peptide-polymer (**7**) was solubilised, side chain deprotected, and separated from insoluble Keiselguhr support by treatment with 95% aq. trifluoroacetic acid (90 min). Deprotection of arginine-containing (**6**) was extended overnight in the presence of phenol (cleavage of the Mtr-group). Both peptide-polymer preparations were freely water soluble; the substance K related polymer eluted at the void volume on gel permeation chromatography (Sephadex G75), suggesting a molecular weight in excess of 50 000.

In these exploratory experiments, fidelity of synthesis was confirmed by ammonolysis of the soluble peptide-polymer at the carboxy terminal ester linkage, gel filtration from the linear polymer, and h.p.l.c. characterisation and amino acid analysis [found: Arg, 1.01; Pro, 2.04; Lys, 1.02; Glu, 2.10; Phe, 1.94; Gly, 1.04; Leu, 1.00; Nle, 1.02 for (**6**); and His, 0.93; Lys, 0.98; Thr, 0.99; Asp, 1.01; Ser, 0.86; Phe, 0.97; Val, 1.00; Leu, 1.00; Met, 0.94; Gly, 2.01 for (**7**)].

All the peptide-polymer conjugates described above were immunogenic in rabbits. Both the formal and ketal substance P-polymers produced useful antibody titres, better than 1:500 and comparable to those elicited by a conventional substance P-bovine serum albumin conjugate at similar points in the immunisation schedule. Antibodies to substance P were detected using peptide labelled at the amino terminus with ^{125}I-Bolton–Hunter reagent. Antibodies to substance K were detected using the blotting procedure described below.

The peptide-polymer conjugates absorbed onto nitrocellulose membranes were incubated with the peptide antisera. The sites of antibody binding were detected using sheep anti-rabbit IgG antisera and rabbit peroxidase–antiperoxidase complex with diaminobenzidine as the chromogen.[8] This procedure detected antibodies to substance P and substance K in sera from rabbits immunised respectively with the substance P- and substance K-polymers. Less than 1 ng of peptide-polymer bound to nitrocellulose was detectable, and under these conditions the polymer itself gave no response. Blots of the peptide polymer conjugates were also detected by anti-peptide antibodies raised conventionally using the appropriate synthetic peptides coupled to protein.

The general principle in solid phase synthesis of using insoluble gel supports which can then be solubilised and further manipulated in free solution appears to be a valuable one. In applications similar to the above, detachment of efficiently synthesised peptides may not normally be required,

† The soluble part was immunogenic.

‡ Predominant formation of antibodies directed against the free amino terminal region was anticipated in the projected biological investigation, and the polymer-bound C-terminal sequence was therefore modified for ease of synthesis. Formation of the more novel N-terminal antibodies is advantageous in permitting distinction between tachykinins with identical C-terminal sequences.

§ Neither substance P nor substance K is intrinsically immunogenic. Low concentrations of the free peptides normally circulate in the plasma.

¶ Anhydrides were used in place of the non-crystalline esters of Fmoc-*O*-t-butyl-serine and -threonine.

and it provides an exceptionally rapid and simple procedure for generating immunogens. It has particular potential for the economic large scale synthesis of peptide vaccines. We anticipate that it will provide a valuable tool in immunochemical research, particularly in investigating the role of adjuvant 'helper' sequences co-synthesised on the same polymer.

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