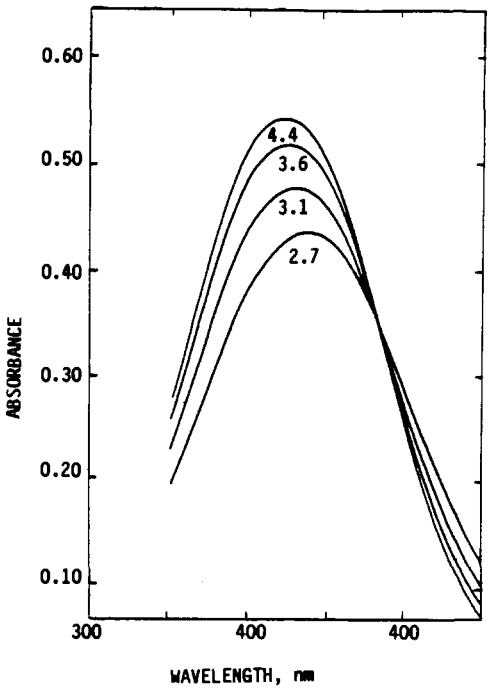

Figure 1. UV-vis spectra of Fe^{III}-H_3THX solutions measured at -log [H^+] values indicated. T_{FeL} = 2.0 × 10^{-4} M, t = 25.0 °C in CH_3OH-H_2O, 1:4 (v/v).

46.38; H, 7.18; N, 5.80. Found: C, 46.48; H, 7.22; N, 5.48. FAB MS $(M + H)^+$ = 688, yield = 85%).

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Long-Chain Polystyrene-Grafted Polyethylene Film Matrix: A New Support for Solid-Phase Peptide Synthesis[1]

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Current methods for the linear scheme of solid-phase synthesis, producing a single peptide analogue per synthesis, are widely based on the original methodology[2] employing divinylbenzene cross-linked polystyrene beads. We report here preliminary investigations on a new support, namely a polystyrene-grafted polyethylene film, that is particularly well-suited for peptide synthesis in a parallel fashion, permitting the simultaneous production of

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    70
Boc-Ala-Asp(OBzl)-Lys(2Cl-Z )-Ala-Asp(OBzl)-

    76
Val-Asp(OBzl)-Val-Leu-Thr(Bzl)-Lys(2Cl-Z)-

                    84
Ala-Lys(2Cl-Z)-Ser(Bzl)-Gln-OCH2-C6H4-CH2-CO-


NH-CH2-polystyrene-grafted polyethylene film
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Figure 1. Protection scheme for the solid-phase assembly of [Asp^{76}]-hPTH fragment (70–84) on 440 wt % polystyrene-grafted polyethylene film.

a multitude of peptide analogues.[3] The pendant long-chain linear polystyrene graft, having a molecular weight on the order of 10^6, may be more accessible for chemical reactions than the polystyrene chains embedded in the traditionally used beads where the molecular weight between cross-links is on the order of 10^4. Both the coupling efficiency during the synthesis and the yield and purity of the final products were comparable to those normally obtained by using the traditional polystyrene bead matrices. The grafted film was prepared by γ-irradiation of polyethylene film placed in a methanolic solution of styrene monomer.[4] The presence of methanol during the grafting process serves the purpose of reducing the swelling of the polystyrene-grafted polyethylene and hence reducing the mobility of the growing polystyrene chains. This effect tends to slow down the diffusion-controlled chain-termination processes and is essential to obtain very long-chain grafts and a high level of chain initiation. In 1972 Tregear reported the use of polystyrene that was 10 wt % radiation-grafted onto Kel F particles for solid-phase synthesis.[5] The polystyrene–Kel F graft support was handled in the same way as the traditional polystyrene beads, in contrast with the new support which can be treated or manipulated as sheets, which can be readily separated from one another.

Preparation of the Matrix. A low-density non-cross-linked polyethylene sheet (0.29 g, 54 μm thickness, 55 cm^2) was γ-irradiated in a sealed ampoule containing approximately 20 mL of thoroughly degassed 30% (v/v) styrene in methanol solution. After irradiation at a dose rate of 400 Gy/h in a ^{60}Co source for 10 h at room temperature the ampoule was left overnight. The film was then extracted in a Soxhlet apparatus with dichloromethane for 96 h and dried to afford a polyethylene film grafted with 1.28 g of polystyrene, i.e., 440 wt % grafting. The weight-average molecular weight (M_w) of the extracted styrene homopolymer, which is also formed during irradiation, was determined by size exclusion chromatography (SEC) to be ca. 2 × 10^6 g/mol, and the ratio of weight-average to number-average molecular weight (M_w/M_n) to be 3. As the styrene homopolymer and the polystyrene grafts are formed under conditions that differ only in the initiation step, these numbers are taken as a measure of the molecular weight characteristics of the polystyrene grafts. Further support for this interpretation was obtained by molecular weight characterization of both the original polyethylene film and the grafted film. For the original film the values M_w = 4 × 10^4 g/mol and M_w/M_n = 5 are obtained by high temperature SEC in 1,2,4-trichlorobenzene. The grafted film is soluble in hot xylene, and SEC was run in this solvent at 90 °C to obtain M_w = 6 × 10^6 g/mol and M_w/M_n = 2, which are similar to the values for the styrene homopolymer by product. The molecular weight of both the polyethylene and the polystyrene-grafted polyethylene were estimated relative to polystyrene standards. The solubility

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(2) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149. For a recent review of the solid-phase technique, see: Barany, G.; Kneib-Cordonier, N.; Mullen, D. G. *Int. J. Peptide Protein Res.* **1987**, *30*, 705.

(3) In the parallel synthesis of multiple peptides, each peptide analogue is synthesized on a labeled sheet of grafted film. The common steps of deprotection, neutralization, washings, and coupling of identical amino acids are performed in a single reaction vessel, while the coupling of different amino acids are carried out in separate reaction vessels. Some recent examples of similar approaches include the following: (a) Geysen, H. M.; Meloen, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3998. (b) Houghten, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 5131.

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Table I. Quantitative Ninhydrin Monitoring[a] of the Solid-Phase Synthesis of Protected Human Parathyroid Hormone Fragment (70–84) on 440 wt % Polystyrene-Grafted Polyethylene Film

	coupling		deprotection	
redidue coupled	remaining free amino groups (μmol/g)	estimated[c] % completn	measd substitutn (mmol/g)	theoretcl substitutn (mmol/g)
84 BocGlnX[d]	0.0	100	0.76 ± 0.02	0.78
83 BocSer(Bzl)	38.0	94.0	0.35 ± 0.04	0.69
82 BocLys(2Cl-Z)	1.6	99.7	0.54 ± 0.03	0.57
81 BocAla	0.6	99.9	0.52 ± 0.03	0.55
80 BocLys(2Cl-Z)	1.2	99.7	0.53 ± 0.02	0.47
79 BocThr(Bzl)	0.0	100	0.44 ± 0.03	0.43
78 BocLeu	0.4	99.9	0.39 ± 0.01	0.41
77 BocVal	0.0	100	0.39 ± 0.03	0.40
76 BocAsp(OBzl)	0.0	100	0.35 ± 0.01	0.37
75 BocVal	0.2	99.9	0.31 ± 0.02	0.35
74 BocAsp(OBzl)	0.7	99.8	0.31 ± 0.02	0.33
73 BocAla	0.0	100	0.29 ± 0.01	0.33
72 BocLys(2Cl-Z)	1.1	99.6	0.30 ± 0.02	0.30
71 BocAsp(OBzl)	1.3	99.5	0.28 ± 0.02	0.28
70 BocAla	0.0	100	0.23 ± 0.01	0.27

[a] Average values based on 2–4 ninhydrin analyses after coupling and deprotection in each cycle expressed as mmol/g of peptide–film. [b] No residues were recoupled but coupling of Boc-Ser(Bzl) was followed by a complete acetylation of remaining free amino groups by using *N*-acetylimidazole in methylene chloride. [c] These estimated values are calculated relative to the theoretical substitution after coupling of the Boc-protected residue and do not include correction for incomplete coupling of the preceding residue. [d] X = $-OCH_2-C_6H_4-CH_2-COOH$.

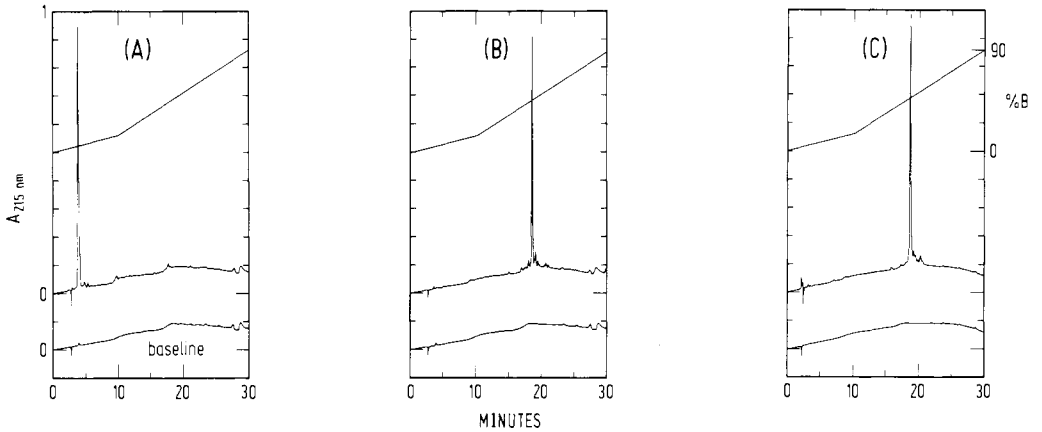

Figure 2. Analytical HPLC chromatograms of (A) crude H-Lys-Ala-Lys-Ser-Gln-OH, (B) crude H-Val-Asp-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln-OH, and (C) crude H-Ala-Asp-Lys-Ala-Asp-Val-Asp-Val-Leu-Thr-Lys-Ala-Lys-Ser-Gln-OH on μBONDAPAK C_{18} (300 × 3.9 mm, 10 μm). Buffer A, H_2O/0.095% CF_3COOH; buffer B, 90% acetonitrile/10% H_2O/0.072% CF_3COOH: flow rate, 1.3 mL/min.

data indicate that there was no significant degree of cross-linking in these linear graft polymers.

Peptide Synthesis. The grafted film was functionalized and tested as a carrier to support the synthesis of the 15-residue C-terminal fragment of human parathyroid hormone hPTH (70–84). Upon aminomethylation,[6] quantitative ninhydrin analysis[7] indicated the substitution of amino groups on the film to be 1.00 mmol/g, and elemental analysis indicated the substitution of nitrogen to be 1.07 mmol/g corresponding to the aminomethylation of one out of every 9.4 phenyl groups (ca. 80% of the matrix is polystyrene). Boc-Gln was loaded onto the aminomethylated film via a preformed Pam handle strategy,[8] and the fully protected film-bound hPTH (70–84), shown in Figure 1, was assembled on 800 mg of film (0.58 mmol Gln) in a manually operated reaction vessel following a standard solid-phase procedure employing coupling with the usual 0.05 M preformed symmetrical anhydrides (1.74 mmol in 35 mL of 20% (v/v) DMF in CH_2Cl_2).[9] As can be seen from the ninhydrin data in Table I, the single coupling of all residues but one proceeded with an efficiency of ≥99.5% (uncorrected for background). Following removal of the N-terminal Boc group, deprotection and release of the peptide from the film support was accomplished with anhydrous HF containing anisol (9:1, v/v) at 0 °C for 1 h. Extractions with ether to remove anisole and alkylkated anisoles from the residual peptide–film mixture were followed by extraction of the peptide into 10% aqueous acetic acid and lyophilization. The hPTH (80–84) and (75–84) fragments were released from the film in the same way. Figure 2 shows HPLC chromatograms of the three unpurified peptides. The homogeneity and overall synthetic yield (ca. 85%, based on quantitative amino acid analyses) are comparable to those obtained by conventional solid-phase synthesis on beads. In the case of hPTH (70–84), 26 mg of pure peptide was obtained from 96 mg of peptide–film. The identity of the peptides was also verified by fast atom bombardment mass spectrometry.

(6) Mitchell, A. R.; Kent, S. B. H.; Erickson, B. W.; Merrifield, R. B. *Tetrahedron Lett.* **1976**, 3795.

(7) Sarin, V. K.; Kent, S. B.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147.

(8) Tam, J. P.; Kent, S. B.; Wong, T. W.; Merrifield, R. B. *Synthesis* **1979**, 955. Preformed 1-hydroxybenzotriazole ester of Boc-Gln 4-(oxymethyl)-phenylacetic acid (4 equiv, 0.08 M) was coupled for 2 h in 30 mL of *N,N*-dimethylformamide/CH_2Cl_2 (1:2, v/v) to give quantitative acylation of the aminomethyl-film.

(9) Synthetic protocol for coupling of Boc-Ser(Bzl), Boc-Ala, Boc-Lys(2Cl-Z), Boc-Thr(Bzl), Boc-Leu, Boc-Val, and Boc-Asp(OBzl): (1) CH_2Cl_2, 3 × 1 min; (2) CF_3COOH/CH_2Cl_2 (1:1, v/v), 3 × 1 min; (3) CF_3COOH/CH_2Cl_2 (1:1, v/v), 1 × 30 min; (4) CH_2Cl_2, 6 × 1 min; (5) *N,N*-diisopropylethylamine/CH_2Cl_2 (1:19, v/v), 3 × 2 min; (6) CH_2Cl_2, 6 × 1 min; (7) 3–10-mg samples were cut off for ninhydrin analyses; (8) protected amino acid was coupled as preformed symmetrical anhydride (3 equiv, 0.05 M) in 35 mL of *N,N*-dimethylformamide/CH_2Cl_2 (1:4, v/v) with shaking for 2 h; (9) CH_2Cl_2, 4 × 2 min; (10) 3–10-mg samples were cut off and neutralized by repeating (5) and (6) for ninhydrin analyses.

The efficient synthesis of hPTH (70-84) on the new support will allow its application on rapid and parallel synthesis of multiple peptide analogues, and we are now completing such a synthesis of melittin analogues using labeled sheets of 285 wt % long-chain polystyrene-grafted polyethylene film.

Acknowledgment. We thank the Danish Biotechnology Program, the Danish Natural Science Research Council, and USPHS CA 36544 for the partial support of this research.

Registry No. hPTH, 9002-64-8; hPTH (80-84), 122425-31-4; hPTH (75-84), 122443-10-1; hPTH (70-84), 98151-37-2; Boc-Gln-$OCH_2C_6H_4$-*p*-CH_2COOH, 83714-72-1; BOC-Ser(Bzl)-OH, 23680-31-1; BOC-Lys(2Cl-Z)-OH, 54613-99-9; BOC-Ala-OH, 15761-38-3; BOC-Thr(Bzl)-OH, 15260-10-3; BOC-Leu-OH, 13139-15-6; BOC-Val-OH, 13734-41-3; BOC-Asp(OBzl)-OH, 7536-58-5; polystyrene-grafted polyethylene, 106826-12-4.

Free Energy Relationships of Substrate and Solvent Hydrophobicities with Enzymatic Catalysis in Organic Media

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While there is little question that enzymes can function in nonaqueous media,[1] the effects of organic solvents on the catalytic activity and substrate specificity of enzymic catalysis are not well understood. Relatively few kinetic studies have been carried out to date.[2] Such quantitative analyses are crucial for the development of kinetic models that can be used to predict optimal solvent and substrate choices for enzymatic reactions in organic media. In the present study, we have examined horseradish peroxidase catalyzed oxidation of phenols in organic solvents as a model to elucidate solvent-induced kinetic alterations of enzymatic catalysis, particularly with respect to substrate and solvent hydrophobicities. Peroxidase is an ideal enzyme for this study as it catalyzes identical reactions in aqueous and organic solvents,[3] and phenols are highly soluble in organic media. In all cases, the enzymatic reactions take place in monophasic organic solutions[5] with diffusional limitations eliminated by adsorption of the peroxidase onto nonporous glass beads.[6]

Our experimental strategy was to determine the steady-state kinetic constants, V_{max}, K_m, and V_{max}/K_m in water and a variety of organic solvents with varying degrees of hydrophobicity.[7] Phenols with para substitutions that differed in hydrophobicities were employed.[9] Such peroxidase substrates do not induce steric hindrance around the phenolic moiety and are similar in electronic factors.[10a,b] Parts A and B of Figure 1 depict the dependence of catalytic efficiency (V_{max}/K_m) on the substrate hydrophobicity, π, in aqueous buffer and several nonaqueous solvents. The catalytic efficiency was profoundly lower (up to 4 orders of mag-

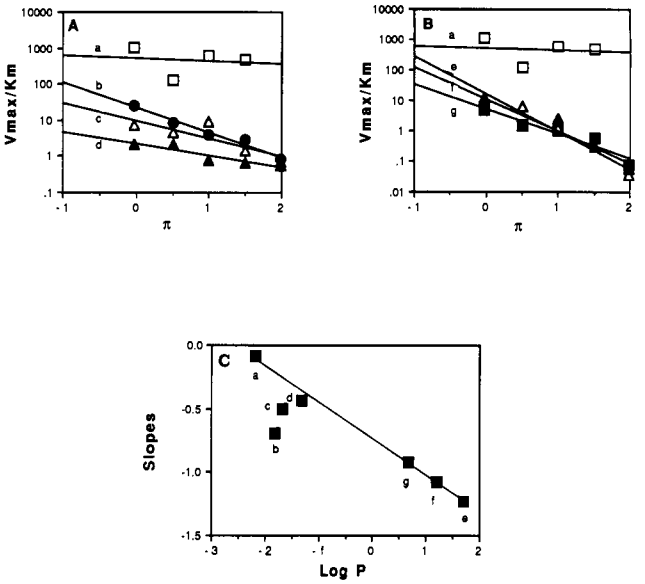

Figure 1. Catalytic efficiency of peroxidase in aqueous and nonaqueous media. (A) Water-miscible solvents: (a) aqueous buffer; (b) dioxane with 30% aqueous buffer; (c) dioxane with 20% aqueous buffer; (d) dioxane with 5% aqueous buffer. (B) Water-immiscible solvents: (a) aqueous buffer; (e) butyl acetate, containing 1% aqueous buffer; (f) propyl acetate, containing 1.5% aqueous buffer; (g) ethyl acetate, containing 2% aqueous buffer. (C) Slopes of (V_{max}/K_m vs π) vs log P. Values of log P for dioxane–water mixtures were calculated by using the following correlation: $\log P = (1 - x) \log P_{solvent} + x \log P_{water}$, where x is the mole fraction of water in the mixture (Reslow, M.; Aldercreutz, P.; Mattiasson, B. *Appl. Microbiol. Biotechnol.* **1987**, *26*, 1-8). Conditions: (i) For phenol oxidation in water, phenol concentrations were varied from 0.05 to 5 mM, the H_2O_2 concentration was fixed at 0.25 mM, and the concentration of peroxidase was 0.01 μg/mL, pH 7.0 (10 mM phosphate buffer), 25 °C. The poor solubility of *p-tert*-butylphenol in aqueous solutions made accurate measurements of kinetic constants impossible. (ii) For phenol oxidation in organic media, phenol concentrations were varied from 10 mM to 1 M, the H_2O_2 concentration was fixed at 0.25 mM, and peroxidase concentrations were in the range from 0.05 to 0.5 μg/mL, 25 °C, shaken at 200 rpm. All kinetic analyses were carried out spectrofluorometrically by following the initial rate of fluorescent dimer production. Kinetic constants were determined by using Eadie–Hofstee graphical representation of duplicate reactions with linear regression analysis. For alkylphenols, excitation and emission were 305 and 350 nm, respectively, while for *p*-methoxyphenol, excitation and emission were 320 and 375 nm, respectively. Correlation of fluorescence intensity with phenol oxidation was carried out by HPLC as described earlier.[2a] The units of V_{max}/K_m are mM^{-1} s^{-1} (peroxidase molecular weight of 42 000[4]).

(1) For general reviews, see: (a) Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194-211. (b) Klibanov, A. M. *Trends Biochem. Sci. (Pers. Ed.)* **1989**, *14*, 141-144. (c) Zaks, A.; Russell, A. J. *J. Biotechnol.* **1988**, *8*, 259-270. (d) Butler, L. G. *Enzyme Microb. Technol.* **1979**, *1*, 253-259. (e) *Biocatalysis in Organic Media*; Laane, C., Tramper, J., Lilly, M. D., Eds.; Elsevier: Amsterdam, 1987.

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(3) In the presence of hydrogen peroxide, horseradish peroxidase catalyzes the single-electron oxidation of phenols and aromatic amines.[4] The reaction mechanism is independent of solvent, and it is possible to compare, directly, the kinetics of phenol oxidation in aqueous and organic media.[2a]

(4) Saunders, B. C.; Holmes-Siedle, A. G.; Stark, B. P. *Peroxidase*; Butterworth: London, 1964.

(5) Defined as enzymatic reactions in the absence of a bulk aqueous phase.[1] This includes water-immisible solvents with water contents below saturation and water-miscible solvents with water contents insufficient to cause enzyme solubilization.

(6) Peroxidase was deposited onto 75-150-μm nonporous glass beads (Sigma Chemical Co.) at a loading of 0.1 mg/g of beads.[2a]

(7) Solvents used ranged in hydrophobicities from water (log P = -2.17) to butyl acetate (log P = 1.70); log P is defined as the logarithm of the partition coefficient between 1-octanol and water (Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 81-87). Added water was 0-30% (v/v) in dioxane, 0% in dimethylformamide and acetone, 2% in ethyl acetate, 1.5% in propyl acetate, and 1% in butyl acetate. In the water-immiscible ester solvents, the water contents were slightly below saturation levels, an amount proven to be optimal for similar solvent systems[8] and confirmed with peroxidase in this work.

(8) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 8017-8021.

(9) Phenolic substituents in the para positions and their respective π values (in parentheses)[10c] included methoxy (-0.02), methyl (0.56), ethyl (1.02), propyl (1.53), and *tert*-butyl (1.98).

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