Organic Process Research & Development **2010,** *14,* 1313–1325

Organic Solvent Nanofiltration: A New Paradigm in Peptide Synthesis

Sheung So,[†] Ludmila G. Peeva,[†] Edward W. Tate,[‡] Robin J. Leatherbarrow,[‡] and Andrew G. Livingston*,[†]

Department of Chemical Engineering and Department of Chemistry, Imperial College London, London SW7 2AZ, U.K.

Abstract:

Solid-phase synthesis is the dominant paradigm for peptide synthesis, used ubiquitously from discovery to production scale. However, the solid-phase approach produces coupling steps that may not be quantitative, introducing errors in amino acid sequences. It also entails an excess of reagents to overcome mass transfer limitations and restrictions on solvent, coupling chemistry, and protecting groups. Organic solvent nanofiltration (OSN) is a newly emerging technology capable of molecular separations in organic solvents. This contribution reports a new technology platform which advantageously combines OSN with solution-phase peptide synthesis, Membrane Enhanced Peptide Synthesis (MEPS). A first amino acid is linked to a soluble polyethylene glycol anchor. Through subsequent repeated coupling and deprotection steps, the peptide is extended to the desired length. The residual byproducts and excess reagents after each reaction are removed by diafiltration through a solvent-stable membrane which retains the peptide. Two pentapeptides are produced using this new technology. The purity of the peptides produced by MEPS is higher than that of peptides produced by solid-phase synthesis, under the same conditions. This illustrates clearly that MEPS benefits from the advantages of solution-phase synthesis, while avoiding the purification steps that have until now made solution-phase synthesis practically difficult.

1. Introduction

Even with the global economic downturn, the market for therapeutic peptides continues to grow and is estimated at about 7.5% annual growth rate for 2009, with a predicted value of more than $13.4 billion by 2010.[1] As of 2009 more than 60 synthetic therapeutic peptides (comprising those used for medical diagnostics or imaging), have reached the American, European, and/or Japanese pharmaceutical markets through a marketing authorization as active pharmaceutical ingredients (APIs).[2] Significantly, large-scale peptide manufacture faces important challenges related to molecular separation in between reaction cycles and final product purification. With solid-phase synthesis established as the dominant paradigm, in this report we draw renewed attention to a process for solution-phase peptide synthesis coupled to separation via organic solvent nanofiltration (OSN).

The idea of applying membrane separation to peptide synthesis has been mainly restricted to reconcentration of peptides produced by biosynthesis[3] or amino acid recovery from aqueous solutions.[4,5] There has been just a single report on using membrane separation for purification between reaction cycles during chemical peptide synthesis performed in organic solvent.[6,7] Peptides were built on poly(ethylene glycol) (PEG) as an anchoring group, and separation of the growing peptide chain from impurities was achieved with ultrafiltration. Since there were no stable membranes in the organic solvents used as reaction media for peptide synthesis, the separation required evaporation of the organic solvent after each coupling and deprotection step, followed by water uptake and neutralisation before ultrafiltration from an aqueous solution. Water was then removed by evaporation and/or azeotropic distillation before redissolving the PEG-peptide back into the organic solvent for the next coupling step. This complex process limited the synthesis to volatile organic solvents, and the lengthy solvent-switching system made the separation process cumbersome—it never found its way to a large-scale peptide synthesis.

Nevertheless, the concept of membrane separation to purify an anchored peptide in between reaction cycles during solution-phase synthesis offers major advantages over solid-phase peptide synthesis (SPPS).[6] The advantages of "classical" solution-phase synthesis are combined with the elegant separation of the solid-phase method. Reaction in the solution phase provides faster reaction rate,[8,9] is less affected by steric hindrance due to peptide folding or reactions within confined spaces which result in transpeptidation, and is not limited by the intraparticle diffusional mass transfer phenomena that can adversely affect SPPS. In terms of industrial utility, solution-phase reactions are easier to scale up due to the absence of swelling effects and cake formation within solid resins that require more complex reactor design.[10]

In addition, the choice of solvents in SPPS is limited to those which swell the peptide—resin matrix,[11] and large reactant excesses (typically up to 5 equiv)[12,13] are usually employed to compensate for mass transfer limitations, thus increasing the

* Corresponding author. Fax: +44 (0)20 75945629. Telephone: +44 (0)20 75945582. E-mail: a.livingston@imperial.ac.uk.

[†] Department of Chemical Engineering.

[‡] Department of Chemistry.

(1) Glaser, V. *Genet. Eng. Biotechnol. News* **2009**, *29*, 1–4.

(2) Vlieghe, P.; Lisowski, V.; Martinez, J.; Khrestchatisk, M. *Drug Discovery Today* **2010**, *15*, 40–56.

(3) Tsuru, T.; Nakao, S.; Kimura, S.; Shutou, T. *Sep. Sci. Technol.* **1994**, *29*, 971–984.

(4) Li, S.-L.; Li, C.; Lui, Y.-S.; Wang, X.-L.; Cao, Z.-A. *J. Membr. Sci.* **2003**, *222*, 191–201.

(5) Wang, X.; Ying, A.; Wang, W. *J. Membr. Sci.* **2002**, *196*, 59–67.

(6) Bayer, E.; Mutter, M. *Nature* **1972**, *237*, 512–513.

(7) Bayer; E.; Gillessen, D.; Kuenzi, H.; Mutter, M.; Studer, R. A process for the manufacture of peptides. (F. Hoffmann-La Roche & Co.)., DE-F2047413, 1980.

(8) Bayer, E.; Mutter, M.; Uhmann, R.; Polster, J.; Mauser, H. *J. Am. Chem. Soc.* **1974**, *96*, 7333–7336.

(9) Gut, V.; Rudinger, J. *Peptides 1968*; E. Bricas: Amsterdam, 1968; pp 185−188.

(10) Edelstein, M.; Scott, P. E.; Sherlund, M.; Hansen, A. L.; Hughes, J. L. *Chem. Eng. Sci.* **1986**, *41*, 617–624.

(11) Fields, G.; Fields, C. *J. Am. Chem. Soc.* **1991**, *113*, 4202.

(12) Amblard, M.; Fehrentz, J.; Martinez, J.; Subra, G. *Mol. Biotechnol.* **2006**, *33*, 239–254.

10.1021/op1001403
Published on Web 10/26/2010

production cost. The type of resin utilised also depends on the first amino acid attached,[14] the length of peptide sequence, and the strategy required, all of which introduce further restrictions to SPPS.

Molecular separation in organic solvents via nanofiltration (OSN) is a fast-emerging technology, with many successful examples so far at laboratory scale.[15−17] It provides gentle separation for heat-sensitive or fragile molecules and does not suffer limitations such as azeotrope formation. Peptide molecules are relatively fragile, sensitive to heat, and synthesized in organic solvents. OSN offers a perfect separation method for the in-cycle purification of growing peptide chains.

This contribution demonstrates a new technology platform that advantageously combines OSN with solution-phase peptide synthesis, Membrane Enhanced Peptide Synthesis (MEPS).[18]

2. Experimental Section

2.1. Materials. All Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt), 4-hydroxymethylphenoxyacetic acid (HMPA), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU) activators, and Wang resin (HMP resin, 100−200 mesh) were purchased from Merck Biosciences, Novabiochem (Switzerland). Polyethylene glycol monomethyl ether (MeO-PEG, MW≈ 5000 g·mol^{-1}), *p*-toluenesulfonyl chloride, potassium phthalimide, hydrazine hydrate, and pyridine used for methylated amino PEG synthesis; *N*,*N*′-diisopropylcarbodiimide (DIC), piperidine, trifluoroacetic acid (TFA), and diisoproyl ethyl amine (DIPEA) required for peptide synthesis; and thymopentin (TP5) standard were supplied by Sigma-Aldrich (UK). Peptide-synthesis grade dimethylformamide (DMF) solvent purchased from Rathburn Chemicals Ltd. (UK) was used in both peptide-chain assembly and diafiltration washes. GPR-grade dichloromethane (DCM), diethyl ether, and ethanol used in methylated amino PEG synthesis were supplied from VWR (UK). Acidolysis solution used for deprotection was made up from phenol/water/TFA (0.7/1/10 in w/v/v ratio).

Three membranes were tested in this primary investigation: (i) a ceramic OSN membrane, the Inopor ZrO_2-coated membrane with 3-nm pore size (MWCO of 2000 g·mol^{-1} as provided by manufacturer Inocermic, Germany) and hydrophobically modified surface; (ii) polymeric SelRO MPF-50 (MWCO 700 g·mol^{-1}, given by the manufacturer, Koch/SelRO) and; (iii) a developmental polyimide (PI) membrane DuraMem1000 (MWCO > 1000 g·mol^{-1}) kindly provided by Evonik Membrane Extraction Technology Ltd., UK.

2.2. Analysis. *Ultraviolet (UV) Spectrometry.* The concentration of all protected amino acids and activators for the rejection experiments was measured in the corresponding solution using Shimadzu UV-2101 PC UV spectrometer, scanned between 200−350 nm.

Gel Permeation Chromatography (GPC). Rejection of PEG and piperidine as well as PEG-peptide losses between processing steps were measured with a Waters GPC system equipped with both Gilson 132 refractive index (RI) detector and a Waters 996 Photodiode Array detector scanning between 250−300 nm. A Waters Styragel HT2 GPC column was used with *N*-methyl pyrrolidone (NMP) solvent as mobile phase, operating at a constant flow rate of 0.5 mL·min $^{-1}$, at 100 °C. The yield (polymer loading) of the MeO-PEG-peptide products was calculated according to eq 1.

$$\text{yield}_{\text{PL}} = \left(\frac{\text{mol of peptide}}{\text{mol of amino-PEG}}\right) \times 100 \qquad (1)$$

Nuclear Magnetic Resonance (NMR). The two-dimensional spectrum of MeO-PEG-HMPA and MeO-PEG-HMPA-Tyr-Fmoc were recorded at 298 K at 400 MHz with a Bruker DRX-400 spectrometer and analysed with MestRe-C software. The loading of HMPA linker and first amino acid were estimated by integrating the aromatic moiety on the HMPA linker (6.7, d), (6.9, d) and Fmoc-protection group (7.7, d) (7.5, d) (7.3, t) (7.2, t) against the reference MeO-group on MeO-PEG (3.4, s).

High Performance Liquid Chromatography (HPLC). The final purity of peptide product and the level of residual impurities in the postreaction mixture during diafiltration washes were measured with an Agilent HPLC system. A reverse-phase HPLC column (ACE C-18, 250 mm ×4.6 mm) packed with 5 μm diameter silica particles with 300 Å pores size was operated at 30 °C. Water and acetonitrile (AcCN) with 0.1% trifluoroacetic acid (TFA) were used as mobile phase, at 1 mL·min^{-1} flow rate. A ramp from 0% AcCN to 80% AcCN in 30 min was followed by 5 min at 100% AcCN and 5 min at 0% AcCN to wash the HPLC column after each run. UV detection was used as a detection method with wavelength set at 210 nm.

Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry. The molecular weight of peptide products was measured by a Bruker Reflex IV MALDI-TOF mass spectrometer. Infrared ionisation was used with ionisation power set at 20 kV. α-Cyano-4-hydroxycinnamic acid was used as the matrix in acetonitrile and ethanol (1:1 ratio) with 0.1% trifluoroacetic acid.

2.3. MEPS Experimental Procedure. The concept of Membrane Enhanced Peptide Synthesis (MEPS) is illustrated in Figure 1. Peptide chain assembly occurs via (1) the coupling step; (2) a washing step for removal of excess reagents via constant volume diafiltration; (3) the deprotection step; (4) a washing step for removal of deprotection byproducts and excess reagents. The cycle is repeated as many times as necessary, adding a further amino acid each cycle, until the desired peptide sequence is obtained. In contrast to the previously reported processes,[6,7] the washing is carried out immediately after the coupling and deprotection steps using the reaction solvent and does not require any solvent exchanges.

The MEPS experimental procedure and setup is depicted in Figure 2. Both coupling and deprotection steps are performed

(13) Chan, W.; White, P. *Fmoc solid phase peptide synthesis: a practical approach*; Oxford University Press: Oxford, 2000.
(14) Barlos, K.; Gatos, D.; Kapolos, S.; Poulos, C.; Schafer, W.; Wenqing, Y. *Int. J. Pept. Protein Res.* **1991**, *38*, 555–561.
(15) Livingston, A.; Peeva, L.; Han, S. J.; Nair, D.; Luthra, S. S.; White, L. S.; Freitas Dos Santos, L. M. *Ann. N.Y. Acad. Sci.* **2003**, *984*, 123–141.
(16) Wong, H. T.; See-Toh, Y. H.; Ferreira, F. C.; Crook, R.; Livingston, A. G. *Chem. Commun.* **2006**, *19*, 2063–2065.
(17) Wong, H. T.; Pink, C. J.; Ferreira, F. C.; Livingston, A. G. *Green Chem.* **2006**, *8*, 373–379.
(18) So, S.; Peeva, L. G.; Tate, E. W.; Leatherbarrow, R. J.; Livingston, A. G. *Chem. Commun.* **2010**, *46*, 2808–2810.

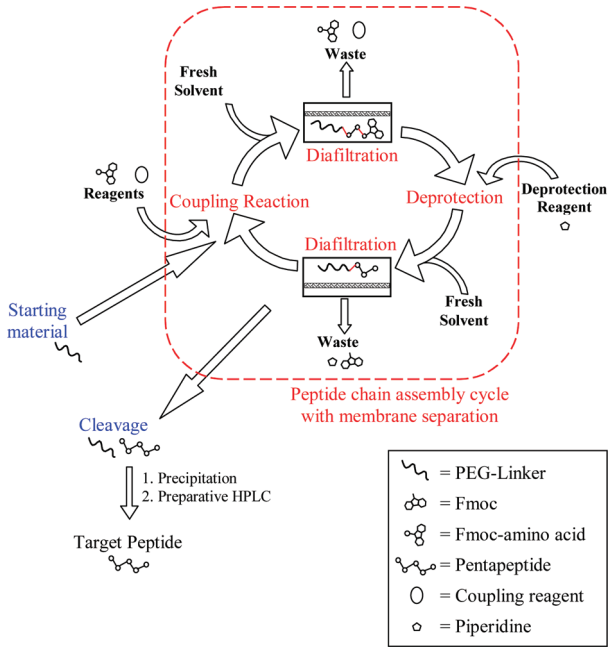

Figure 1. **Schematic representation of the membrane enhanced peptide synthesis (MEPS) process. Peptide chain assembly cycle was performed using the apparatus presented in Figure 2.[18] (Reproduced by permission of The Royal Society of Chemistry http://dx.doi.org/10.1039/b926747f.)**

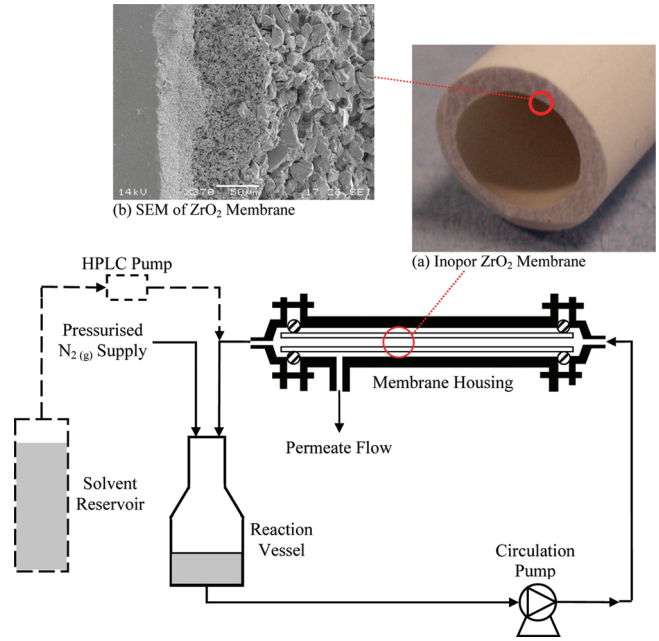

Figure 2. **Experimental setup used to obtain rejection measurements and perform peptide chain assembly. (i) Solid lines represent the recirculation loop and permeate flow used for batch rejection measurements. A quantity of 200 mL of test solution was charged into the reaction vessel, and 100 mL of permeate was collected for analysis. (ii) Dashed lines represent the additional equipment required for diafiltration after each reaction during peptide chain assembly. Both coupling and deprotection reactions were performed in the reaction vessel where mixing was provided via the circulation pump. Upon completion of each reaction, the system was pressurised to 7 bar gauge using nitrogen gas. The resulting permeate flow rate was measured using a measuring cylinder. Fresh DMF solvent was pumped via an HPLC pump from the solvent reservoir into the system to replace the permeated solvent and maintain constant liquid volume within the reaction vessel; (a) Inopor ZrO_2 ceramic membrane - front view; (b) SEM image of the Inopor ZrO_2 ceramic membrane - edge view at magnification 370×. The line bar corresponds to 50 μm.[18] (Reproduced by permission of The Royal Society of Chemistry http://dx.doi.org/10.1039/b926747f.)**

in the reaction vessel at atmospheric pressure. The circulation pump recirculates the reaction solution (~200 mL) through the membrane cartridge and ensures good liquid mixing throughout. Upon completion of each reaction, the system is pressurised using N_2 to ~7 bar gauge. The resulting solvent flow permeating through the membrane is balanced by a constant flow of fresh solvent (DMF) supplied to the reaction vessel from the solvent reservoir via an HPLC pump. The same procedure is applied at each reaction/washing cycle. The peptide is assembled on a soluble polymeric support to increase retention by the membrane. PEG was chosen as the polymeric support in the MEPS process due to its good solubility in solvents used in peptide synthesis, excellent chemical stability, low toxicity, its availability in different sizes and shapes, and low cost. It has also been shown that the PEG anchoring group has only a minor influence upon the peptide conformation in various solvents, but considerably improves peptide solubility.[19] Due to these major advantages the liquid-phase synthesis on PEG has been largely investigated in the past and successfully applied in the field of peptides,[20] oligonucleotides[21,22] and oligosaccharides production as well as for combinatorial library synthesis.[23−25] In addition, PEGylated peptides and proteins (peptide/protein with covalently attached PEG) are receiving much attention recently as a new generation of drugs with enhanced therapeutic and pharmacokinetic potential,[26−28] and so it might be possible to utilise the product peptide without cleavage from the PEG.

Methoxy-amino-PEG with molecular weight (MW) 5000 $g \cdot mol^{-1}$ (MeO-PEG-NH_2) was used in this study. PEGs with higher molecular weights would improve membrane retention of peptide product but may also reduce reactivity[8,29] and increase solution viscosity, the latest typically resulting in lower solvent flux through the membrane.[30] A linker (HMPA) was first attached to the MeO-PEG-NH_2, to ensure facile cleavage of the final peptide via acidolysis. In the case of PEGylated peptide

(19) Toniolo, C.; Bonora, G. M.; Mutter, M. *J. Am. Chem. Soc.* **1979**, *101*, 450–454.
(20) Fischer, P.; Zheleva, D. *J. Pept. Sci.* **2002**, *8*, 529–542.
(21) Drioli, S.; Adamo, I.; Ballico, M.; Morvan, F.; Bonora, G. M. *Eur. J. Org. Chem.* **2002**, 3473–3480.
(22) Bonora, G. M.; Rossin, R.; Zaramella, S.; Cole, D. L.; Eleuteri, A.; Ravikumar, V. T. *Org. Process Res. Dev.* **2000**, *4*, 225–231.
(23) Wentworth, P., Jr. *Trends Biotechnol* **1999**, *17*, 448–452.
(24) Gravert, D. J.; Janda, K. D. *Curr. Opin. Chem. Biol.* **1997**, *1*, 107–113.
(25) Gravert, D. J.; Janda, K. D. *Chem. Rev.* **1997**, *97*, 489–509.
(26) Basu, A.; Yang, K.; Wang, M.; Liu, S.; Chintala, R.; Palm, T.; Zhao, H.; Peng, P.; Wu, D.; Zhang, Z.; Hua, J.; Hsieh, M.-C.; Zhou, J.; Petti, G.; Li, X.; Janjua, A.; Mendez, M.; Liu, J.; Longley, C.; Zhang, Z.; Mehlig, M.; Borowski, V.; Viswanathan, M.; Filpula, D. *Bioconjugate Chem.* **2006**, *17*, 618–630.
(27) Lee, S.-H.; Lee, S.; Youn, Y. S.; Na, D. H.; Chae, S. Y.; Byun, Y.; Lee, K. C. *Bioconjugate Chem.* **2005**, *16*, 377–382.
(28) Roberts, M.; Bentley, M.; Harris, J. *Adv. Drug Delivery Rev.* **2002**, *54*, 459–476.
(29) Akula, K. C.; Boddy, K.; Doma, U. *Indian J. Chem.* **2006**, *45B*, 678–683.
(30) Vandezande, P.; Gevers, L.; Vankelecom, I. *Chem. Soc. Rev.* **2008**, *37*, 365–405.

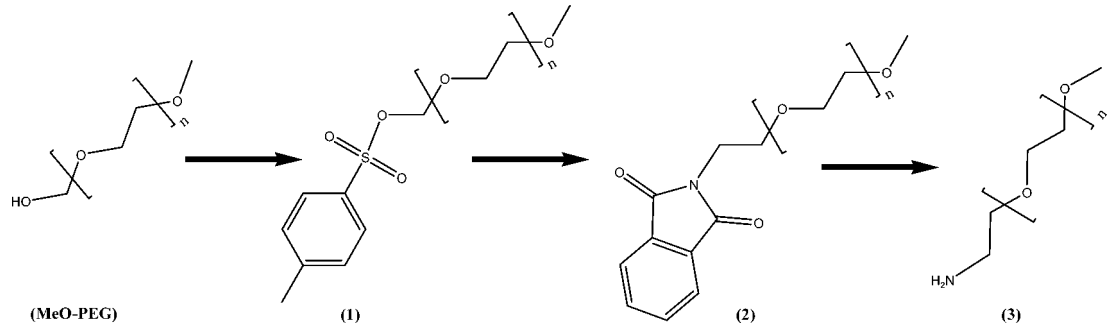

Figure 3. **Methylated amino PEG synthesis proposed by Pillai et al.[32]**

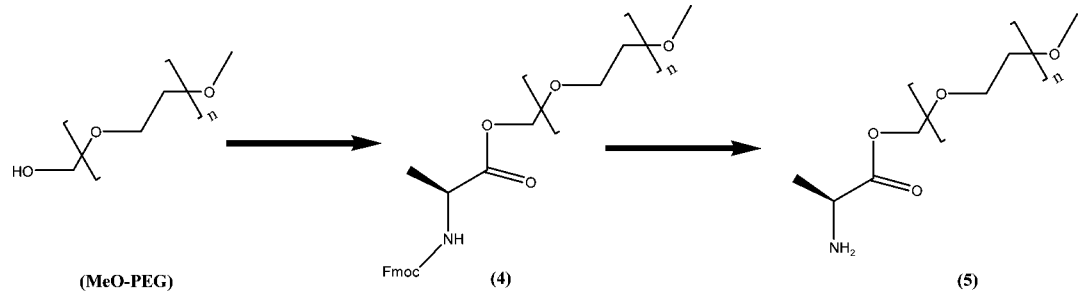

Figure 4. **Methylated amino PEG synthesis developed in our laboratory**

synthesis the use of a linker would not be necessary. Fmoc (9-fluorenylmethoxycarbonyl) chemistry was chosen for the peptide chain assembly due to its widespread application and mild deprotection conditions utilising piperidine. It is known that the peptide coupling reaction performs best in polar aprotic solvents such as dimethylformamide (DMF) or *N*-methyl pyrrolidinone (NMP), and DMF was used in both reaction and diafiltration steps. Until recently, the use of these solvents in combination with OSN membranes would not have been possible; however, recently developed solvent-stable membranes[31] possess good stability in these polar aprotic solvents and make this technology achievable.

The rejection tests for the Inopor ZrO_2 membrane were performed at 7 bar gauge in batch mode using the setup presented in Figure 2. 200 mL of test solution was charged into the Reaction Vessel and 100 mL of permeate was collected for analysis. The rejection tests for polymeric membranes were performed in a Sepa ST dead-end filtration cell (Osmonics, USA) in batch mode. 50 mL of test solution was charged to the cell and 25 mL permeate collected for analysis. The rejection tests for MPF50 membrane were performed at 30 bar gauge while the experiments with DuraMem1000 were performed at 5 bar gauge. All experiments were performed at ambient temperature.

2.4. Synthesis Procedures. *Methylated Amino-poly(ethylene glycol) (MeO-PEG-NH_2) Synthesis.* Two methods were used to produce MeO-PEG-NH_2, a procedure proposed by Pillai et al.[32] (Figure 3) and a procedure developed in our laboratory, (Figure 4).

MeO-PEG-Tosylate Synthesis (1). Polyethylene glycol monomethyl ether (MeO-PEG, MW∼5000 g·mol^{-1}) was dehydrated *in vacuo* at 80 °C for 4 h before dissolving in DCM (25 mL per mmol MeO-PEG). *p*-Toluenesulfonyl chloride (46 mmol per mmol MeO-PEG) and pyridine (1.5 mL per mmol PEG) were added to the PEG solution, and reaction was performed under nitrogen atmosphere and continuous stirring for 12 h. The resulting solution was concentrated *in vacuo*, and the product (MeO-PEG-Tos) was precipitated by adding diethyl ether and kept at 4 °C for few hours to complete the precipitation. The precipitate was filtered and washed with ether, recrystallised with ethanol, and dried *in vacuo*. UV analysis was performed by measuring the absorption at 270 nm to verify the presence of the tosylate group.

MeO-PEG-Phthalimide Synthesis (2). MeO-PEG-Tos and potassium phthalimide (10 mmol per mmol MeO-PEG-Tos) were dissolved in DMF (14 mL per mmol MeO-PEG-Tos), and heated under reflux and nitrogen atmosphere for 4 h. Residual solids were removed by filtration, and diethyl ether was added to filtrate to precipitate product from the solution. The resulting slurry was kept at 4 °C for a few hours to complete the precipitation. The product was filtered and washed with ether, followed by digestion with DCM. The insoluble impurities were filtered and MeO-PEG-Phth was precipitated from filtrate with ether. The solid product was filtered again, washed with ether and dried *in vacuo*. The appearance of the phthalimide group was verified by UV analysis at 292 nm and 264 nm.

MeO-Amino-PEG synthesis (3). MeO-PEG-Phth and hydrazine hydrate (40 mmol per mmol MeO-PEG-Phth) were dissolved in ethanol (18.5 mL per mmol MeO-PEG-Phth) and heated under reflux for 12 h. The product mixture was cooled to room temperature before precipitation with diethyl ether. The precipitate was filtered and redissolved in DCM, the insoluble impurities were filtered and MeO-PEG-NH_2 product was precipitated from the filtrate, washed with diethyl ether, recrystallised with ethanol and finally dried *in vacuo*. The product was analysed with UV analysis for disappearance of the phthalimide group and the Kaiser test used to verify the presence of amino groups. The conversion (∼80%) was determined by titration with $HCl_{(aq)}$.

(31) Toh, Y. S.; Lim, F.; Livingston, A. *J. Membr. Sci.* **2007**, *301*, 3–10.
(32) Pillai, V.; Mutter, M.; Bayer, E.; Gatfield, I. *J. Org. Chem.* **1980**, *45*, 5364–5370.

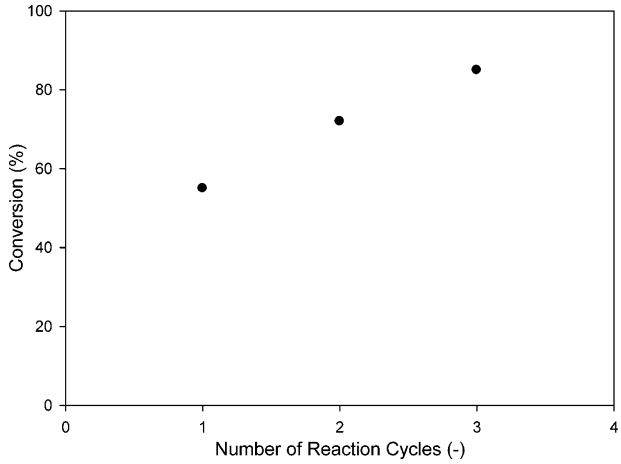

Figure 5. **Reaction conversion for Fmoc-Ala attachment onto MeO-PEG as a function of the number of reaction cycles performed. Conversion >80% is achieved after three coupling reaction cycles.**

Attachment of Fmoc-Ala onto MeO-PEG (4). MeO-PEG was dissolved in DCM (10 mL per mol MeO-PEG), while Fmoc-Ala, HOBt (both 2 mol per mol MeO-PEG), and DIPEA (1 mol per mol MeO-PEG) were dissolved in DMF (4 mL per mol MeO-PEG). DIC (2 mol per mol MeO-PEG) was added afterward to the DMF solution and preactivated for 15 min before the two solutions were mixed together. The resulting solution was mixed vigorously for 2 h at 4 °C. Solid impurities were filtered, and the product was precipitated with diethyl ether and dried. The coupling step was repeated three times to obtain conversion >80%, Figure 5. MeO-PEG-Ala-Fmoc product was precipitated first from DMF by adding diethyl ether, followed by recrystallisation with ethanol. The conversion was determined with ^{1}H NMR as a ratio between the peaks at 3.4 (s, 3H) for the MeO group and 1.4 (d, 1H) for the Me group of alanine.

Deprotection of Fmoc Group (5). Standard 20% v/v piperidine/DMF solution was used to remove the Fmoc-protecting group from (**4**). After deprotection the product was precipitated and washed with diethyl ether, recrystallised with ethanol, and dried *in vacuo*. ^{1}H NMR was used to verify the disappearance of the Fmoc-group at 7.2 (t, 2H), 7.3 (t, 2H), 7.5 (d, 2H), and 7.7 (d, 2H), and the Kaiser test was used to confirm the presence of amino functional groups.

Peptide Synthesis. The peptide synthesis procedure developed by Fischer et al.[20] was modified by incorporating membrane separation steps.

Synthesis of MeO-PEG-HMPA. MeO-PEG-NH_2 (**3**) or (**5**) was dissolved in DCM. 4-Hydroxymethylphenoxyacetic acid (HMPA), PyBOP (both 2 mol per mol MeO-PEG-NH_2) and DIPEA (1 mol per mol MeO-PEG-NH_2) were preactivated in DMF for 15 min before being added into the PEG solution and mixed vigorously for 2 h. The product was precipitated with diethyl ether at 4 °C for 2 h and separated by centrifugation, followed by ether washes. The crude product was purified by recrystallisation with ethanol. MeO-PEG-HMPA product was dried *in vacuo* and analysed by ^{1}H NMR analysis. Conversion was estimated on the basis of the ratio between peaks at 3.4 (s, 3H) for the MeO-group and 6.7 (d, 2H), 6.9 (d, 2H) for the aromatic system on the HMPA linker.

Synthesis of Fmoc-aa$_{(1)}$-HMPA-PEG-OMe for MEPS and SPPS. For the MEPS process, MeO-PEG-HMPA was predissolved in DMF (45 L per mol MeO-PEG-HMPA). Fmoc-protected amino acid (*Fmoc-aa*$_{(1)}$), HOBt, DIC (all 2 mol per mol MeO-PEG-HMPA) and DIPEA (1 mol per mol MeO-PEG-HMPA) were preactivated for 15 min in DMF (10 L per mol MeO-PEG-HMPA) before being mixed with MeO-PEG-HMPA solution for 1 h. Upon reaction completion the excess reagents were removed by constant volume diafiltration (refer to Figure 2 for details). Permeate samples were collected to monitor any PEG-peptide losses and to verify the removal of impurities. Small retentate samples were collected and precipitated for ^{1}H NMR analysis to estimate the conversion, and the Kaiser test was used to confirm the absence of amino groups. For the SPPS process, Wang resin was preswollen in DMF (3 volumes per bed volume) for 30 min followed by drainage of the excess solvent. Fmoc-protected amino acid (*Fmoc-aa*$_{(1)}$), HOBt, DIC (all 2 mol per kg Wang resin), and DIPEA (1 mol per kg Wang resin) were preactivated for 15 min in DMF (2 volumes per bed volume) before being added to the wet resin. The resulting mixture was shaken vigorously for 1 h, followed by microfiltration and washes (5 × 2 volumes per bed volume).

Chain Assembly with Fmoc-Amino Acid for SPPS and MEPS. For the MEPS process, Fmoc-amino acid was preactivated with PyBOP, HOBt (all 2 mol per mol MeO-PEG-HMPA) and DIPEA (1 mol per mol MeO-PEG-HMPA) in DMF (10 L per mol MeO-PEG-HMPA) for 15 min. A separate solution of MeO-PEG-HMPA-*aa*$_{(1)}$-H in DMF was prepared and mixed with the preactivated solution. The resulting solution was mixed vigorously for 1 h followed by diafiltration (10 volumes per starting volume). For the SPPS process, Fmoc-amino acid was preactivated with PyBOP, HOBt (all 2 mol per kg Wang resin), and DIPEA (1 mol per kg Wang resin) in DMF (2 volumes per bed volume) for 15 min. The solution was added to the swollen Wang resin, and the resulting mixture was shaken vigorously for 1 h, followed by microfiltration and washes (5 × 2 volumes per bed volume).

Fmoc-Deprotection for SPPS and MEPS. For the MEPS process, 20% piperidine/DMF solution was prepared by adding the required amount of pure piperidine to the known solution volume within the reaction vessel (see Figure 2). Deprotection was performed for 30 min. Purification after deprotection was performed via diafiltration (12 volumes per starting volume). For the SPPS process, a standard 20% piperidine in DMF solution was premixed and added to the peptide-resin (2 volumes per bed volume). The mixture was shaken vigorously for 30 min, followed by microfiltration and washes (6 × 2 volumes per bed volume).

Side Chain Deprotection and Cleavage Reaction for SPPS and MEPS. For the MEPS process, the solution containing PEG-peptide building block was removed from the reaction vessel, and the product was precipitated with diethyl ether and dried *in vacuo*. The precipitate was then redissolved into 20 mL per mmol of acidolysis solution for 3 h. Diethyl ether was used to precipitate the target peptide together with MeO-PEG-HMPA. For the SPPS process, peptide-resin building block was gradually switched from DMF to diethyl ether via methanol and DCM and dried *in vacuo*. Acidolysis solution was mixed vigorously

Table 1. **Rejection data for PEGs, protected amino acids, and other common reagents used in peptide synthesis obtained with an Inopor ZrO_2 ceramic membrane with 3-nm pores and the polymeric membrane DuraMem1000[a]**

				Inopor ZrO_2 membrane		DuraMem1000 membrane	
entry	compound	MW [g·mol^{-1}]	nature	rejection [%]	mass balance error[b] [%]	rejection [%]	mass balance error[b] [%]
1	Fmoc-Ala-OH	311	amino acid	55	7.3	31	15
2	Fmoc-Arg $(Boc)_2$-OH	597	amino acid	68	8.7	−	
3	Fmoc-Asp (O^tBu)-OH	412	amino acid	41	9.2	−	−
4	Fmoc-Cys (^{t}Bu)-OH	400	amino acid	50	3.8	−	−
5	Fmoc-Gly-OH	297	amino acid	51	13.5	−	−
6	Fmoc-Lys (Boc)-OH	469	amino acid	59	8.7	66	12
7	Fmoc-Trp (Boc)-OH	527	amino acid	61	3.6	−	−
8	Fmoc-Tyr (^{t}Bu)-OH	460	amino acid	41	6.6	44	1
9	Fmoc-Val-OH	339	amino acid	42	4.5	−	−
10	DIC	126	activator	13	45.5	6	3
11	HOBt	135	racemization suppressor	32	7.3	22	3
12	HOBt·H_2O	153	racemization suppressor	61	12.4	−	−
13	HBTU	379	activator	51	7.0	−	−
14	PyBOP	520	activator	64	3.5	63	19
15	HMPA	182	linker	39	11.4	−	−
16	piperidine	85	deprotection reagent	5	3.3	0.5	12
17	MeO-PEG	5000	anchor	99	9.7	99	8
18	MeO-PEG-HMPA	5182	anchor	99	39.8	−	−

[a] The experiments were performed in a batch mode, at ambient temperature and the rejection was determined according to eq 2. The rejection tests for the Inopor ZrO_2 membrane were performed at 7 bar gauge and at 5 bar gauge for the DuraMem1000. [b] Error estimated from a mass balance between feed, permeate, and retentate.

with the solid resin for 3 h. Liquid was filtered from the solid resin, and peptide was precipitated from the filtrate with diethyl ether. In both processes a small amount of the final peptide was separated by semi-preparative RP-HPLC for characterization purposes. Analytical RP-HPLC and MALDI mass spectrometry were performed to determine peptide purity and molecular weight.

3. Results and Discussion

3.1. Membrane Selection. The most important part for successful realisation of the process is the choice of membrane. The membrane selected should possess a number of specific characteristics: (1) Excellent long-term stability in the reaction solvent, DMF; the process also involves continuous switching of the reaction media between the DMF solution in the coupling step and 20% piperidine/DMF solution in the deprotection step, and membrane performance should not be affected by these changes; (2) high selectivity between MeO-PEG-peptide, and side-reaction products and excess reagents such as unreacted amino acids, activators, and deprotection reagents. To minimize product losses it is desirable that the membrane rejection (eq 2) of the MeO-PEG-peptide is as close to 100% as possible. On the other hand, the removal of "waste" reagents is extremely important in order to avoid "random sequencing" caused by unreacted amino acid or deprotection reagent which is not washed out from the system and carries over to the subsequent coupling step. Thus, the rejection of the "waste" reagents should be sufficiently low to prevent excessive wash solvent volumes which could make the MEPS process attractive.

$$\text{rejection of species } i, \quad R_i\,(\%) = \left(1 - \frac{C_P^i}{C_R^i}\right) \times 100\% \qquad (2)$$

where C_P^i is the concentration of solute i at the permeate side of the membrane and C_R^i is the concentration of solute i at the retentate side of the membrane.

The membrane selection strategy for the MEPS process was first, to investigate the stability of a membrane in the processing solvent, DMF, and deprotection reagent, 20% v/v piperidine/DMF; second, for stable membranes, to investigate the rejection of the MeO-PEG anchoring group by those membranes; and third, for membranes fulfilling the first two criteria, to measure rejection of the reagents used in peptide synthesis, e.g., amino acids, activators, and deprotecting reagents. Due to the early stage of OSN development the choice of commercially available organic solvent-stable membranes is quite limited. According to our knowledge of the membrane market, there are currently five companies producing solvent-resistant nanofiltration membranes. The commercially available solvent-stable membranes were preselected for this study on the sole basis of the manufacturer information. These membranes include the Koch/SelRO MPF50 membrane, the newly launched DuraMem membrane, and the Inopor ceramic membrane (see section 2.1). Since ceramic-based membranes could be expected to have better compatibility and more stable performance in DMF, the Inopor ceramic membrane was investigated first. The Inopor ceramic membrane showed no obvious changes when soaked in DMF or 20% piperidine/DMF solution for a week, suggesting good (at least short-term) stability. Rejection data for this membrane are presented in Table 1.

The Inopor membrane exhibited 99% rejection of both MeO-PEG and MeO-PEG-HMPA (entries 17 and 18, Table 1). Simple calculations show that even 99% rejection would result in approximately 10% loss of product when 10 volumes of diafiltration solvent are used for washing 1 volume of reaction

solution. However, during the course of peptide synthesis the rejection of the MeO-PEG-peptide is expected to increase up to ~100% due to the increasing molecular weight of the growing peptide chain and its bulkier structure, and so in fact product losses will be considerably less.

The membrane rejection of a number of Fmoc-amino acids was determined, covering a wide range of properties—the lowest MW amino acid and the highest MW amino acid, acidic, basic, and hydrophobic amino acids, and some of the amino acids most frequently occurring in proteins (entries 1−9, Table 1). An interesting observation from these data is that the rejection of the protected amino acids is governed by the character of the protecting group. For the Fmoc-protected amino acids without Boc side-chain protection, the rejections are between 40−50%, while those amino acids containing Fmoc as a main-chain protecting group and Boc as a side-chain protecting group exhibit rejections within the range of 60−70%. The rejection increases by +10% per Boc group regardless of the nature of the amino acids. A probable explanation is that these bulky and hydrophobic protecting groups impede transport through the OSN membrane. The relationship between solute structure and its permeability through the membrane has been studied by many researchers.[33−38] In general the radius of a molecule affects its diffusivity across the membrane—more bulky molecules pass through the membrane more slowly and hence exhibit higher rejection. Steric hindrance caused by larger or more branched substituents is known to be one of the major factors affecting rejection of a compound, while interactions between solute and membrane based on hydrophobicity[35−37] or charge also play a major role in mass transfer across the membrane. This result is quite different from the results reported in the literature for nanofiltration of biosynthetic broth, where the rejection of amino acid strongly depends on the nature of the amino acid—acidic, basic, hydrophobic, etc.[39,40] In aqueous systems, the pH-dependent ionisation of acidic and basic amino acids apparently has a larger role in determining rejection.

Further rejection tests on coupling activators and reagents were performed. Entries 10−14 in Table 1 show that PyBOP and HBTU activators exhibit relatively high rejection, presumably due to the fact that these activators are bulky salts so that both steric hindrance and Donnan effects would contribute to their retention.[38] Fortunately, during the coupling reaction these activators break down into smaller molecules; hence, the rejection from postreaction solutions is expected to be considerably lower. The coupling reagent DIC showed very low rejection of 13%, similarly, only 5% of piperidine, the deprotection reagent for the Fmoc-deprotection, was rejected (entry 16, Table 1). This is expected since both molecules are small and nonbranched, and should permeate easily through the membrane. DIC is used only in combination with HOBt as racemisation suppressor, and so it is important to examine the rejection of HOBt. There are two types of HOBt available on the market. One is dry, crystalline flake HOBt, the other is a wet powder, $HOBt \cdot H_2O$. As shown in entries 11 and 12, the moisture content seems to considerably affect the rejection of HOBt by the membrane. The other two activators, PyBOP and HBTU, do not require addition of HOBt[41] because the latest breaks off as a product from these activators during the coupling reaction. The rejection of the break-off product is expected to be low, since it is free of moisture. Finally the rejection of HMPA linker (entry 15, Table 1) was also investigated in order to evaluate diafiltration as an option to purify MeO-PEG-HMPA after the attachment of linker onto MeO-PEG-NH_2. Although the rejection of HMPA is relatively low (39%) with 99% rejection of MeO-PEG-HMPA, the product losses will still be ~10% during the separation step, and it was decided to purify MeO-PEG-HMPA by a precipitation method (see Experimental Section).

The solvent flux through this membrane was stable, ~27−30 $L \cdot m^{-2} \cdot h^{-1}$. With the membrane area of ~0.011 m^2, a reasonable permeate flow was achieved. On the basis of the rejection data and material balance equations for the diafiltration, a mathematical simulation was performed to estimate the wash solvent volume required for removal of excess reagents and reaction byproducts after each reaction step (Figure 6). This simulation for the coupling reaction was based on 2 equiv of starting reagents per 1 equiv MeO-PEG-HMPA and assumes 100% conversion after each coupling step. As intuitively expected, the two reagents with the highest rejections, the activator PyBOP and the protected Fmoc-Arg$(Boc)_2$ amino acid, require the largest wash solvent volume (~10 volumes per starting volume) for removal to less than 0.05 equiv. Figure 6 suggests that, after 10 volumes of wash solvent per starting volume, the washout becomes less efficient, and since most reagents remaining at this point are below 0.03 equiv, simulations were limited to 10−12 volumes. For the deprotection reaction, despite the high excess of piperidine employed (~40 equiv) due to its low rejection of ~5% (entry 16, Table 1), 10−12 wash solvent volumes should be sufficient for its almost complete removal to less than 0.01 equiv. Other chemicals with low rejection such as DIC and HOBt should also be readily removed with 10 volumes of diafiltration solvent.

The results obtained with the Inopor membrane seemed quite promising. The main drawbacks with using ceramic membranes are their relatively high price and fragile structure. In this respect better choices are their counterparts made from polymeric materials. Unfortunately, the most widely available polymeric OSN membranes on the market from the StarMem family manufactured by W.R. Grace are made of polyimide and are almost instantaneously soluble in DMF. The best commercially available alternatives from the polymeric OSN membranes series seemed to be Koch/SelRO MPF-50 (MWCO 700 $g \cdot mol^{-1}$) (currently not on sale by the manufacturer, but still

(33) Wang, X.-L.; Tsuru, T.; Nakao, S.; Kimura, S. *J. Membr. Sci.* **1997**, *135*, 19–32.
(34) Toon, Y.; Lueptow, R. *J. Membr. Sci.* **2005**, *261*, 76–86.
(35) Kiso, Y.; Sugiura, Y.; Kitao, T.; Nishimura, K. *J. Membr. Sci.* **2001**, *192*, 1–10.
(36) Kiso, Y.; Kon, T.; Kitao, T.; Nishimura, K. *J. Membr. Sci.* **2001**, *182*, 205–214.
(37) Kiso, Y.; Nishimura, Y.; Kitao, T.; Nishimura, K. *J. Membr. Sci.* **2000**, *171*, 229–237.
(38) Donnan, F. *J. Membr. Sci.* **1995**, *100*, 45–55.
(39) Martin-Orue, C.; Bouhallab, S.; Garem, A. *J. Membr. Sci.* **1998**, *142*, 225–233.
(40) Tsuru, T.; Nakao, S.; Kimura, S.; Shutou, T. *Sep. Sci. Technol.* **1994**, *29*, 971–984.
(41) Carpino, L.; El-Faham, A.; Albericio, F. *Tetrahedron Lett.* **1994**, *35*, 2279–2282.

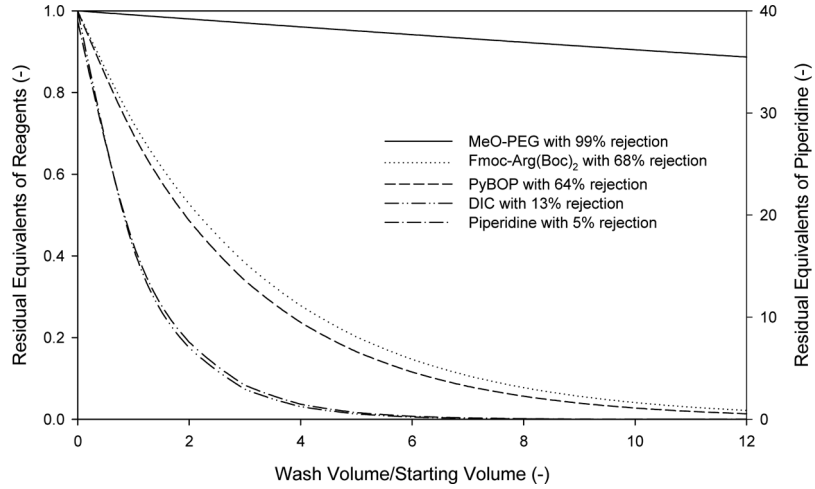

Figure 6. **Estimated residual equivalents of reagents in the postreaction mixture during diafiltration with Inopor ZrO_2 membrane, as a function of the wash solvent volume per starting postreaction mixture volume. The estimate was based on the rejection data for each individual reagent (Table 1) and material balance equations for the diafiltration. For the coupling reaction 2 equiv of reagents per 1 equiv of MeO-PEG-HMPA and 100% conversion are assumed, and for the deprotection 40 equiv of piperidine are assumed.**

in stock in our lab) and the recently launched DuraMem membrane series which are claimed to be stable in aprotic solvents.[42]

The commercial MPF-50 is a composite membrane from the MP series manufactured by Koch Membrane Systems/SelRO. It consists of a PDMS dense top layer and a porous PAN supporting layer.[30] Although this membrane has currently been taken off the market, we decided to investigate its applicability to the MEPS process. Our first observation was that, due to the small membrane area in the dead end cell of 0.0014 m^2 and relatively low DMF flux ($\sim$20 $L \cdot m^{-2} \cdot h^{-1}$), the nanofiltration process was running very slowly. On the basis of the protocol by Fischer et al.[20] who used a mixture of DCM/DMF (1:1 ratio) for the coupling reaction we decided to investigate DCM and the solvent mixture as well. The first and most important observation was that, apart from swelling, there were no other significant changes of the MPF-50 membrane in DCM and the mixed solvent system, suggesting that the membrane has at least good short-term stability. Flux of DCM was about 130 $L \cdot m^{-2} \cdot h^{-1}$ on average, and that of the mixture was 120−130 $L \cdot m^{-2} \cdot h^{-1}$, while that of the DMF alone was only 20 $L \cdot m^{-2} \cdot h^{-1}$ at ambient temperature and 30 bar gauge. This difference in permeation flux could be due to two reasons. First, DMF (0.92 cP at 20 °C) is a more viscous solvent than DCM (0.44 cP at 20 °C), which may decrease the transport across the membrane and lead to a lower flux;[30] second, many polymeric membranes and polymeric matrices are known to swell well in DCM, and the MPF-50 membrane has a PDMS top separation layer that has been known to swell exceptionally well in chlorinated solvents.[43] Swelling experiments revealed a 5.4% expansion of the MPF-50 membrane in DCM, while only 0.9% expansion was measured in DMF. Both phenomena may contribute to the higher permeation fluxes. The rejection of some targeted compounds is illustrated in Table 2. Amino acids containing different N-terminal protection groups in different solvent systems were examined. The results confirmed our previous observation with the Inopor ZrO_2 membrane, that the amino acid transport through the membrane is governed by its protection group and is nearly independent from the properties and MW of the amino acid itself. In general Fmoc-protected amino acids showed the highest rejection in both DCM and DCM/DMF solvent mixture. It was followed by Boc-protected, and the Z-protected amino acid was least retained in the DCM/DMF solvent mixture. The order of the last two was reversed in pure DCM solvent. This could be due to different solvent/solute and/or solvent/membrane interactions or a combination of the three. This is an interesting phenomenon, as the protecting group dominates the properties of the amino acid, the interaction between protecting group and solvent probably

Table 2. **Rejection data for PEGs, protected amino acids, and reagents commonly used in peptide synthesis obtained with MPF-50 polymeric membrane at 30 bar gauge and ambient temperature in DCM, and DCM/DMF 1/1 solvent mixture in batch rejection experiments**

			solvent			
			DCM		DCM/DMF (1/1)	
entry	compound	MW [$g \cdot mol^{-1}$]	rejection [%]	mass balance error[a] [%]	rejection [%]	mass balance error[a] [%]
1	Fmoc-Ala-OH	311			73	3
2	Fmoc-Trp-OH	427	68	21	74	32
3	Fmoc-Tyr-OH	403	64	19	76	17
4	Fmoc-Trp(Boc)-OH	527	–	–	72	13
5	Fmoc-Tyr(^{t}Bu)-OH	460	–	–	73	8
6	Z-Trp-OH	338	59	–	26	–
7	Z-Tyr-OH	315	40	–	57	–
8	Boc-Trp-OH	304	20	–	66	–
9	Boc-Tyr-OH	281	35	–	60	–
10	DIC	126	–	–	24	9
11	HOBt	135	–	–	42	2
12	HBTU	379	–	–	91	8
13	PyBOP	520	–	–	98	10
14	HMPA	182	–	–	65	27
15	piperidine	85	–	–	9[b]	25
18	MeO-PEG	5000	–	–	99	13

[a] Error estimated from a mass balance between feed, permeate, and retentate. [b] Rejection measured in pure DMF.

(42) http://duramem.evonik.com/product/duramem-puramem/en/Pages/default.aspx.

(43) Vankelecom, I.; De Smet, K.; Gevers, L.; Livingston, A.; Nair, D.; Aerts, S.; Kuypers, S.; Jacobs, P. *J. Membr. Sci.* **2004**, *231*, 99–108.

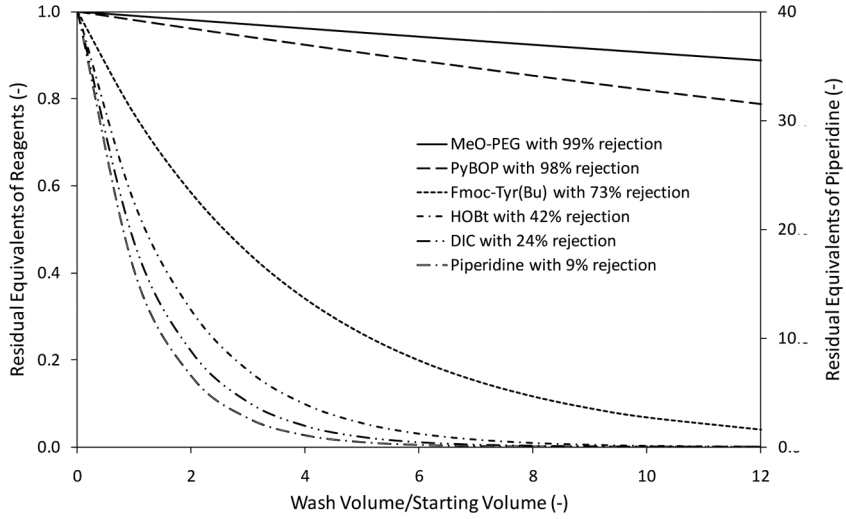

Figure 7. **Estimated residual equivalents of reagents in the postreaction mixture during diafiltration with MPF-50 membrane as a function of the wash solvent volume per volume of the starting postreaction mixture. The estimate was based on the rejection data for each individual reagent (Table 2) and material balance equations for the diafiltration. For the coupling reaction, 2 equiv of reagents per 1 equiv of MeO-PEG-HMPA and 100% conversion are assumed, and for the deprotection 40 equiv of piperidine are assumed.**

also plays an important role in the transport mechanism of the molecule across the membrane. Results also showed that, in general, the rejection of amino acid in pure DCM solvent was lower than that in DCM/DMF mixture. This can be explained by the higher degree of swelling in DCM and lower viscosity compared to those for DMF, which ensures faster diffusion of the solute. The rejections of the Fmoc-protected amino acids were very high, and on the basis of the mathematically simulated curve for the washing volume in Figure 7, it can be concluded that even 10 volumes of wash solvent will only reduce the level of Fmoc-amino acid to ~10% of the initial concentration, which is likely to be too high for the MEPS process. The rejection of the traditional DIC coupling reagent (Table 2, entry 10) and HOBt racemisation suppressor (Table 2, entry 11) were also measured in DCM/DMF (1:1) solvent mixture. Results showed both have low rejection by the MPF-50 membrane which is in agreement with their low MW and the rejection results obtained with the Inopor ZrO_2 membrane. The rejection of coupling reagents, HBTU and PyBOP (entries 12, 13) were both high and close to the rejection of MeO-PEG, suggesting that these coupling reagents are not appropriate for the MEPS process using MPF-50 as separation membrane, since it will be nearly impossible to wash them out without unacceptably high losses of the product (see Figure 7). On the basis of the rejection results and mathematical simulations, it was concluded that MPF-50 is not appropriate for the MEPS process.

The final membrane investigated was from the DuraMem series (developmental DuraMem1000 membrane kindly provided by Evonik Membrane Extraction Technology Ltd.). According to the manufacturer data this membrane made of modified polyimide, is stable in DMF and has a MWCO > 1000 $g \cdot mol^{-1}$. No visible changes were observed in the membrane after 48 h exposure to DMF and 20% piperidine-DMF solution, suggesting at least short-term stability. The DMF solvent flux given by this membrane was very high reaching, 100 $L \cdot m^{-2} \cdot h^{-1}$ at 5 bar gauge, which would allow fast purification of the postreaction mixture. The rejection data for this membrane are placed for easy comparison in Table 1. As can be seen from the table the rejections are very similar to the ones obtained with the Inopor membrane. The rejection of the MeOPEG was 99% (same as for the Inopor membrane), and the rejection of PyBOP which actually determines the wash solvent volume required for purification after the coupling step was also the same. Similar trends for the rejection of the other tested compounds were observed for both membranes. Obviously this makes the DuraMem1000 membrane also a very good candidate for the MEPS process. Finally, we choose the Inopor ZrO_2 membrane to further demonstrate the MEPS process.

3.2. Peptide Synthesis with MEPS Process: Proof of Principal. Two pentapeptide sequences were assembled to demonstrate the MEPS process. To prove the concept we started by producing a model peptide with the simple repeatable sequence Tyr-Ala-Tyr-Ala-Tyr. The sequence was chosen in order to include one of the largest common protected amino acids Fmoc-Tyr(^{t}Bu) and one of the smallest protected hydrophobic amino acids, Fmoc-Ala. Thus, the first synthesis should provide information on the performance of the MEPS process with respect to different molecular sizes and properties of amino acids.

PyBOP was the activator chosen for the coupling reaction because it is known as one of the best activators and, at the same time, is one of the largest of the commercial activators available and thus will be a challenge for the MEPS process. DIC was used for the esterification linking the first amino acid onto MeO-PEG-HMPA and could also be used for the coupling reaction if the cost of PyBOP activator were an issue at industrial scale. Thus, the first synthesis gives insight into how both activators behave during the diafiltration, where their postreaction forms could perform differently from the original compounds.

Finally, this first experiment also sought to answer questions about the membrane stability at high concentrations of organic base (piperidine) during the deprotection step and/or the

Table 3. **Estimated yield, solvent usage, and Kaiser test results at different synthetic/purification steps of pentapeptide synthesis**

	separation method								
	organic solvent nanofiltration			precipitation/filtration[d]			organic solvent nanofiltration		
	H-Tyr-Ala-Tyr-Ala-Tyr-OH						thymopentin (H-Arg-Lys-Asp-Val-Tyr-OH)		
synthetic step	yield[a] [%]	solvent used[b] [L·mol^{-1}]	Kaiser test	yield[a] [%]	solvent used[b] [L·mol^{-1}]	Kaiser test	yield[a] [%]	solvent used[b] [L·mol^{-1}]	Kaiser test
attachment of linker	100	~	+	100	~	+	100	~	−[e]
coupling	99.6	544.8	−	99.6	1645	−	99.2	504	−
deprotection	99.1	657.2	+	99.0	966	+	99.0	611	+
coupling	98.6	550.4	−	82.7	1005	+[e]	98.5	517	−
deprotection	98.4	661.5	+	79.3	1202	+	98.4	630	+
coupling	98.1	553.0	−	70.4	1146	-	98.2	534	−
deprotection	97.9	664.7	+	66.9	1231	+	98.1	653	+
coupling	97.6	555.8	−	58.4	1358	−	98.0	556	−
deprotection	97.5	667.9	+	54.6	1484	+	97.9	681	+
coupling	97.1	558.8	−	47.3	1666	−	97.8	580	+[e]
deprotection	97.0	671.6	+	42.9	1834	+	97.8	713	+
cleavage	54.6[c]	~		~	~		82[c]	~	

[a] Relative mass yield, mass of material retained at the end of given synthetic/purification step with respect to the mass of material after the linker attachment step, estimated by GPC analysis for OSN and dry yield for precipitation/filtration. [b] Solvent usage was estimated on the basis of the assumption of 100% conversion per synthetic step. [c] The process is not optimised, and losses were incurred during the product removal from the nanofiltration system, where considerable amounts of liquid remained in the tubes and the circulation pump head; note that the final product was separated via precipitation/filtration where additional product losses occurred. We believe that, after suitable setup and process optimisation, the whole product could be successfully recovered. [d] The product from the precipitation/filtration process was not cleaved and analysed; we aimed to compare only the solvent consumption. [e] Kaiser test result inconclusive.

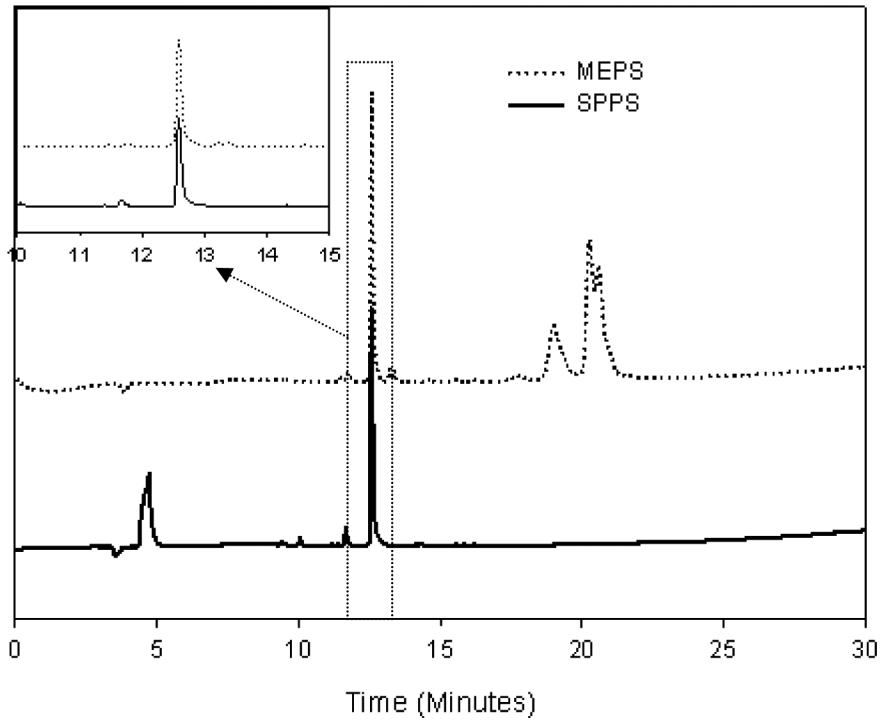

Figure 8. **RP-HPLC chromatograms of H-Tyr-Ala-Tyr-Ala-Tyr-OH produced from the MEPS process and H-Tyr-Ala-Tyr-Ala-Tyr-OH standard produced from the SPPS process. Results showed target peptide produced from MEPS that eluted at 12.5 min contained no detectable amounts of peptide byproduct. The large peaks that eluted between 19−21 min were PEGylated wastes such as MeO-PEG-HMPA. The small peaks that eluted before 12 min and after 13 min had negligible areas, and MALDI-TOF analysis performed did not suggest they are of peptide origin.**

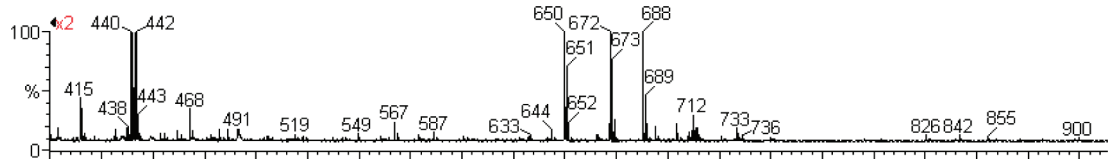

Figure 9. **MALDI-TOF mass spectrum of final model peptide H-Tyr-Ala-Tyr-Ala-Tyr-OH after semi-preparative HPLC purification. Molecular weights 650, 672, and 688 correspond to the target peptide mass, 649 Da, in the ionic form of MH^+, MNa^+, and MK^+, while peaks at 440 and 442 correspond to the matrix itself.**

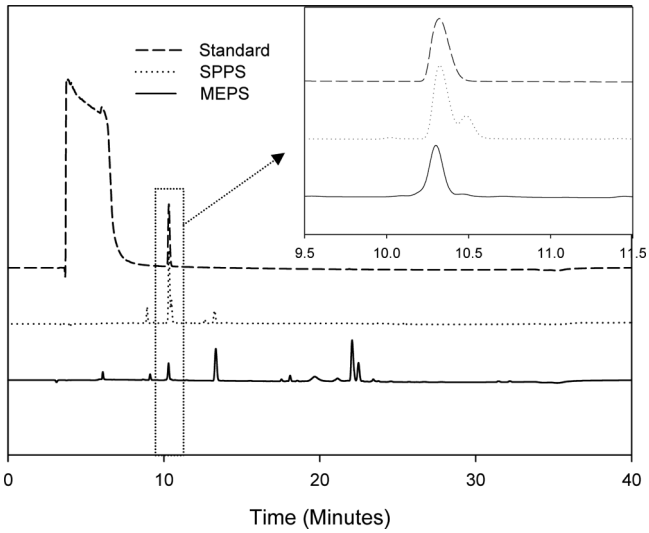

Figure 10. **HPLC chromatograms of peptide TP-5 produced by MEPS and SPPS processes, and TP-5 standard purchased from Sigma-Aldrich (UK). The target TP-5 peptide was eluted at 10.3 min. Both syntheses (MEPS and SPPS) were performed under the same reaction conditions of 2 equiv of reagents per 1 equiv peptide and single reaction cycles. Peptide purity was determined as a ratio between the target peptide TP-5 peak area and the total area of the peaks corresponding to peptide sequences in the solution. The purity of TP-5 produced by MEPS was determined as ~94% (two impurities eluted at 10.0 and 10.4 min), while TP-5 produced by SPPS was ~77% pure (one impurity eluted at 10.5 min). The large peaks eluted between 19−23 min were PEGylated wastes such as MeO-PEG-HMPA, and the peak eluted at 13 min was not of peptide origin as confirmed by MALDI-TOF analysis; these peaks were not taken into account for the purity calculations.[18] (Reproduced by permission of The Royal Society of Chemistry http://dx.doi.org/10.1039/b926747f.)**

potential for membrane fouling due to membrane−peptide or membrane−postreaction mixture interactions.

On the basis of the theoretically estimated values, the solvent volume required for diafiltration was set at 10 wash volumes/starting volume for each coupling reaction and 12 wash volumes/starting volume for each deprotection reaction. The bigger wash volume after deprotection step should almost completely eliminate piperidine residues from the system and prevent undesirable reactions. For comparison we also performed a parallel synthesis where purification was achieved via traditional PEG-peptide precipitation/filtration technique (precipitation with diethyl ether followed by recrystallisation with ethanol, see Experimental Section for details). The solvent consumption and yield obtained after each synthetic/purification step are presented in Table 3. The results seem to be quite favorable for MEPS process; however, since both processes have not been optimised, these results are only preliminary.

As expected, the rejection of the MeO-PEG-peptide increased to >99.7% after the first and the second amino acid attachment and ~100% for later attachments. Permeation fluxes of 27−30 $L \cdot m^{-2} \cdot h^{-1}$ at 7 bar gauge operating pressure were achieved during the washes and were comparable with the fluxes obtained during rejection tests on individual compounds, which suggests no membrane fouling or significant concentration polarisation[44] occurs. This flux is also comparable to the fluxes of other commercially available organic solvent resistant membranes[42,45] and could be increased if higher pressure is applied, making the washing step even more time efficient.

The peptide produced using 2 equiv of reagents per 1 equiv peptide and single reaction cycle was of excellent purity (>99%), confirmed by HPLC and MALDI-TOF analysis (see Figures 8 and 9). The same peptide synthesised as a control on a standard Advanced ChemTech Apex 396 synthesiser using 5 equiv of reagents, also gave ~100% purity (Figure 8). Purity estimations were based on the HPLC results and were obtained as a ratio between the area of the targeted peptide sequence and the total area of peaks corresponding to peptide sequences in the solution. "Waste" products such as MeO-PEG derivatives were not taken into account. We recognise that the PEGylated "waste" could create problems in the crude peptide purification step. One possible solution is to use an alternative anchoring group more easily separable from the peptide via precipitation or membrane separation. The molecular weight of the product was confirmed with a MALDI-TOF mass spectrum shown in Figure 9. The lack of "random sequencing" in the final product proves that the membrane purification is efficient during the whole process and demonstrates the excellent membrane performance and lifetime. While our modelling suggested that traces of impurities would still remain in the system after each purification step, and although this was confirmed by the HPLC analysis, the level of residues did not seem to affect the final product purity. Apparently the "acceptable" level of impurities that can be tolerated in the system without provoking side reactions is higher than anticipated, and a lower volume of washing solvent might be employed.

The overall yield (polymer loading) of H-Tyr-Ala-Tyr-Ala-Tyr-OH produced by the MEPS process was estimated to be ~81%, with respect to the MeO-PEG-NH_2 material (based on the HPLC analysis of the crude peptide and standard produced by the automatic synthesiser). This relatively low yield was due to initial imperfections of our experimental procedure and setup and supposed relatively low conversion at the first synthetic step (attachment of the linker to MeO-PEG-NH_2 estimated from the NMR was somewhat ~80−85%; unfortunately, the spectra were too noisy and fluctuating to obtain accurate estimation). After optimisation and the use of modified anchoring group, the yield of the second peptide synthesised, as will be shown below, considerably improved.

One important element of the proposed MEPS process is the purity of the anchoring group MeO-PEG-NH_2. If the conversion of MeO-PEG to MeO-PEG-NH_2 is not complete, the nonconverted MeO-PEG is carried throughout the whole process as inert material that increases solution viscosity and reduces the filtration efficiency. For the first peptide, to synthesize MeO-PEG-NH_2 we used the method proposed by Pillai et al.[32] who reported a conversion of 80%. This method was found to be highly sensitive to the moisture content of PEG, and the conversion was not robustly reproducible. An alternative method for MeO-PEG-NH_2 production was developed on the

(44) Peeva, L.; Gibbins, E.; Luthra, S.; White, L.; Stateva, R.; Livingston, A. *J. Membr. Sci.* **2004**, *236*, 121–136.
(45) (a) http://www.kochmembrane.com. (b) http://www.sterlitech.com. (c) http://www.inopor.com.

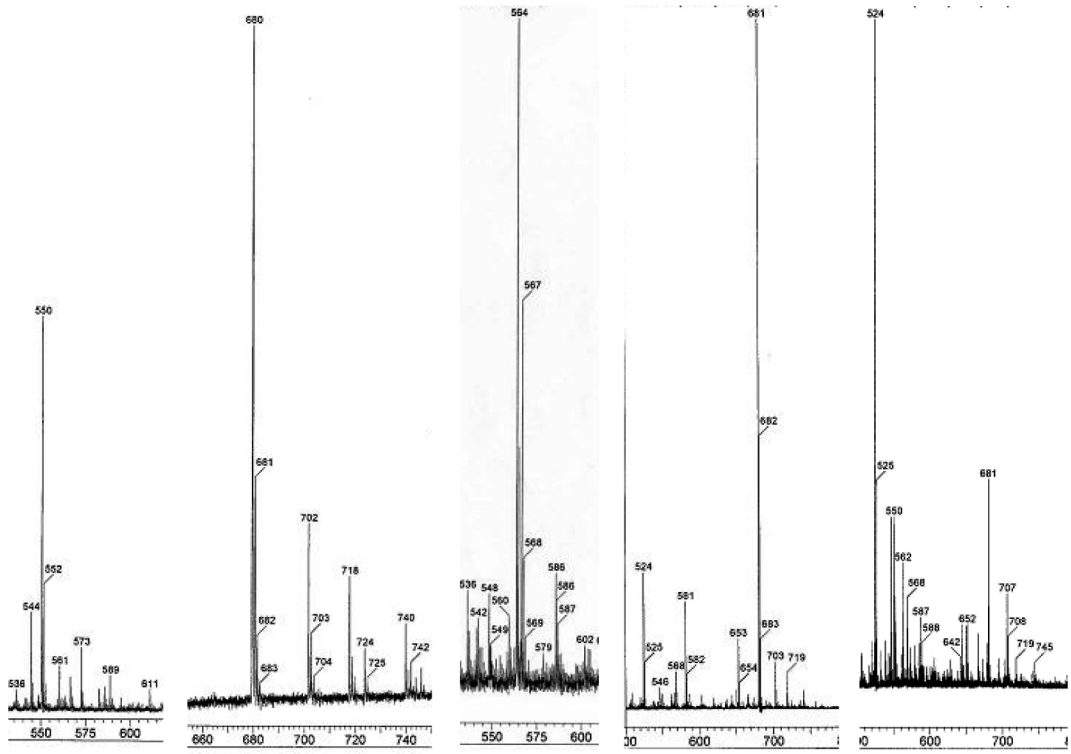

Figure 11. **MALDI-TOF mass spectrum of the different peptides produced by MEPS and SPPS TP5 synthesis and isolated using semi-preparative HPLC. Spectra (B) from the MEPS process and (D) from the SPPS process correspond to the peak eluted at 10.3 min. It was identified as TP5 and showed the target molecular mass, MH^+ of 680 Da. Spectra (A) and (C) from the MEPS process correspond to the two impurities eluted at 10.0 and 10.4 min, respectively, and were identified as deletion of Asp, MH^+ 564 Da and Lys, MH^+ 550 Da. Spectrum (E) from the SPPS process corresponds to the impurity eluted at 10.5 min and was identified as deletion of Arg, MH^+ 524 Da.[18] (Reproduced by permission of The Royal Society of Chemistry http://dx.doi.org/10.1039/b926747f.)**

basis of a simpler reaction procedure: attachment of FmocAla directly onto MeO-PEG. The method is less sensitive to the moisture content of PEG, with reproducible conversion >80% and utilises only reagents used in peptide synthesis (see the Experimental Section for details). This further improved the efficiency of the MEPS process.

Encouraged by our first success and fortified with an improved anchoring group, a second pentapeptide, thymopentin (H-Arg-Lys-Asp-Val-Tyr-OH) was synthesized as a second demonstration of the MEPS process. Thymopentin (TP-5) is a derivative of naturally occurring hormone thymopoietin, which regulates the differentiation and maturation of T cells in the human immune system.[46] Recent studies have shown that this immunomodulator has great potential to treat rheumatoid arthritis, AIDS, and other primary immunodeficiencies. Besides being a potential active pharmaceutical ingredient (API), this sequence covers a variety of amino acids from acidic (Tyr and Asp) and basic (Lys and Arg) amino acids to a hydrophobic amino acid (Val), known to be difficult for coupling.[47] It also contains one of the largest Fmoc-/Boc-protected amino acids (Fmoc-Arg$(Boc)_2$, MW 597 $g \cdot mol^{-1}$), and so this synthesis is a significant challenge for the MEPS process.

The coupling reaction was performed again, using 2 equiv of reagents per 1 equiv of peptide and a single reaction cycle. For comparison the same peptide was synthesised via manual SPPS on a Wang resin under the same reaction conditions (see Experimental Section for details).

HPLC analyses of TP-5 produced by both MEPS and SPPS are illustrated in Figure 10. The purity of the MEPS product was estimated as ~94%. The MALDI-TOF analysis shown in Figure 11 confirmed the product's molecular weight of MH^+ 680. The two impurities (at 10.0 and 10.4 min) were identified as peptides resulting from the deletion of Asp, MH^+ 564, and Lys, MH^+ 550. TP-5, produced by SPPS under the same conditions of 2 equiv reagents per 1 equiv peptide and single reaction cycle, was only 77% pure. The main impurity in SPPS was identified as deletion of Arg, MH^+ 524, as shown in Figure 11. The observed Arg deletion may be due to the fact that, being the biggest protected amino acid used in this work (MW 597 $g \cdot mol^{-1}$), its diffusion into the solid resin was slower which resulted in a lower reaction rate and amino acid deletion, respectively. In the MEPS process, where reaction is performed in solution, this constraint is considerably reduced, and no deletion was observed. We are not sure what caused the deletion of Asp and Lys in the MEPS process. It may have something to do with the interactions between these amino acids and the anchoring group. Although it has been shown that PEG has

(46) Ochoa, E.; Medrano, S.; de Carlin, M.; Dilonardo, A. *Cell. Mol. Neurobiol.* **1988**, *8*, 325–331.
(47) Katakai, R. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 1173–1178.

very little to no effect on the peptide conformation, the presence of a 5000 $g \cdot mol^{-1}$ molecule may still have some influence and partial restriction on the accessibility. Nevertheless the overall result demonstrates the advantage of homogeneous reaction, in terms of a higher purity obtained.

The overall yield (polymer loading) of TP-5 produced by the MEPS process was estimated to be ~92%, with respect to the MeO-PEG-NH_2 material (based on the HPLC analysis of the crude peptide and calibration produced using the TP5 standard from Sigma-Aldrich), which is a considerable improvement from the first synthesis.

4. Conclusions

For this investigation a 1.8 mmol batch of peptide H-Tyr-Ala-Tyr-Ala-Tyr-OH and a 0.9 mmol batch of peptide TP-5 were produced which yielded ~1 g and ~0.6 g of product, respectively. With the current laboratory setup it will be easy to produce 20 mmol batches of peptide by simply increasing the feed volume and using identical operating conditions. Further scale-up to kilogram and even ton scale should be possible by simply increasing the size of the equipment.

The MEPS process proposed in this work integrates the advantages of performing peptide synthesis in solution with a direct membrane purification of the postreaction mixture. The process is easy to scale up, is less constrained by mass transfer limitations, requires a smaller excess of reagents than SPPS, and yet still demonstrates excellent purity and yield of the final peptide. Further optimisation of the separation step and wash solvent volume will result in important solvent savings and improved process economics. Thus, in our opinion, the MEPS process offers an important alternative route for peptide production at industrial scale, especially as a production method for PEGylated peptides. The MEPS process is yet another demonstration of the great potential of OSN as a separation and purification technology in the fine chemicals and pharmaceutical industries.

Acknowledgment

This work was funded by the U.K. Engineering and Physical Sciences Research Council (EPSRC) Grant NoEP/C50982X/1; we acknowledge also the financial support from the FP7 IAPP EC Grant NoPIAP-GA-2008-218068.

LIST OF ABBREVIATIONS FOR REAGENTS AND PROTECTING GROUPS USED IN THE MEPS/SPPS PEPTIDE SYNTHESIS

Compound	Compound Name	Nature
Boc	*tert*-Butoxycarbonyl	Protecting group
DCM	Dichloromethane	Solvent
DIC	*N*,*N*′-Diisopropylcarbodiimide	Activator
DIPEA	Diisopropylethylamine	Base
DMF	Dimethylformamide	Solvent
Fmoc	9-Fluorenylmethoxycarbonyl	Protecting group
Fmoc-Ala-OH	*N*-α-Fmoc-L-alanine	Amino acid
Fmoc-Arg $(Boc)_2$-OH	*N*-α-Fmoc-*N*-ω,*N*-ω′-bis-*tert*-butoxycarbonyl-L-arginine	Amino acid
Fmoc-Asp (O^tBu)-OH	*N*-α-Fmoc-L-aspartic acid β-*tert*-butyl ester	Amino acid
Fmoc-Lys (Boc)-OH	*N*-α-Fmoc-*N*-ε-*tert*-Boc-L-lysine	Amino acid
Fmoc-Tyr (^{t}Bu)-OH	*N*-α-Fmoc-O-*tert*-butyl-L-tyrosine	Amino acid
Fmoc-Val-OH	*N*-α-Fmoc-L-valine	Amino acid
HMPA	4-Hydroxymethylphenoxyacetic acid	Linker
HOBt	1-Hydroxybenzotriazole·H_2O	Racemization suppressor
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate	Activator
Piperidine	Piperidine	Deprotection reagent
MeO-PEG	Methylated polyethylene glycol (methoxypolyethylene glycol)	Anchor
Wang HMP resin	*p*-Benzyloxybenzyl alcohol resin	SPPS resin

Received for review May 20, 2010.

OP1001403