Supporting Information for:

Membrane Enhanced Peptide Synthesis

Experimental Methods

Materials

All Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt), 4-hydroxymethylphenoxyacetic acid (HMPA), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro-phosphate (PyBOP) activator and Wang resin (HMP resin, 100-200 mesh) were purchased from Merck Biosciences, Novabiochem (Switzerland). *p*-Toluenesulfonyl chloride, potassium phthalimide, hydrazine hydrate and pyridine used for methylated amino PEG synthesis and N,N'-diisopropylcarbodiimide (DIC), piperidine, trifluoroacetic acid (TFA) and diisoproyl ethyl amine (DIPEA) required for peptide synthesis 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 solvent washes. GPR graded dichloromethane (DCM), diethyl ether and ethanol used in methylated amino PEG synthesis were supplies from VWR (UK). Acidolysis solution used for deprotection was made up from phenol/water/TFA (0.7/1/10 in w/v/v ratio).

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.* [S1] (**Figure S1**) and a procedure developed in our laboratory, (**Figure S2**).

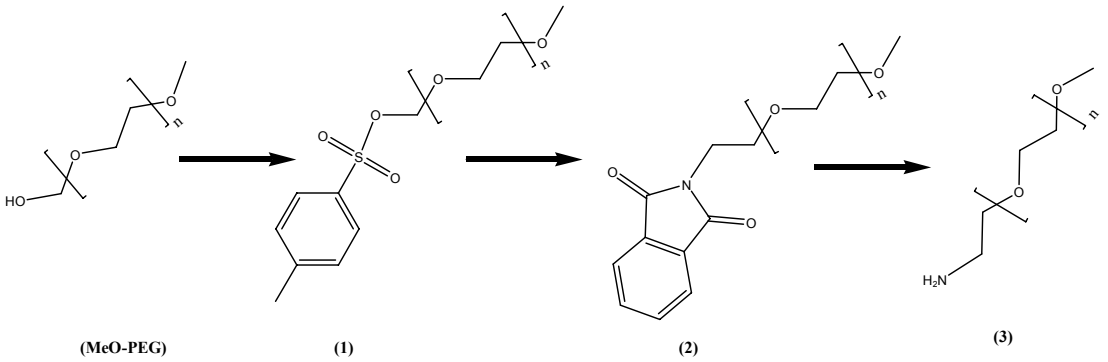

Figure S 1: Methylated amino PEG synthesis proposed by *Pillai et al.* [S1]

***MeO-PEG-Tosylate synthesis* (1)**. Polyethylene glycol monomethyl ether (MeO-PEG, MW~5000 $gmol^{-1}$) was dehydrated *in vacuo* at 80°C for 4 hours 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 hours. 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 270nm 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 hours. 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 292nm and 264nm.

***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 hours. The product mixture was cooled to room temperature before precipitation with diethyl ether. The precipitate was filtered and re-dissolved 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 $HCl_{(aq)}$.

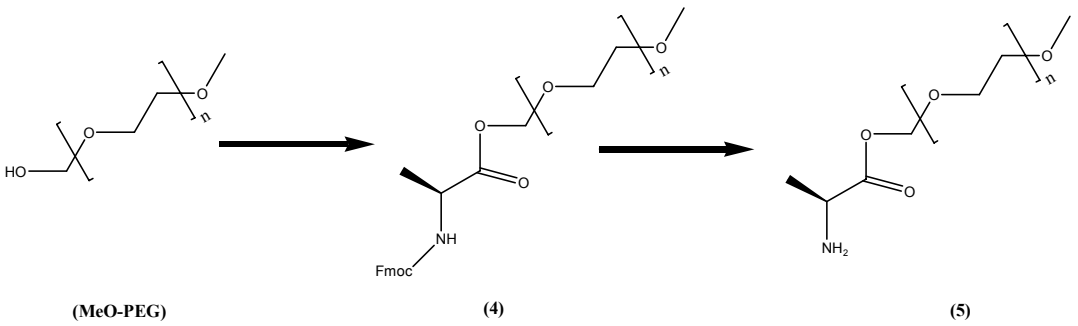

Figure S 2: Methylated amino PEG synthesis developed in our laboratory

***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 pre-activated for 15 minutes before mixing the two solutions together. The resulting solution was mixed vigorously for 2 hours at 4°C. Solid impurities were filtered and the

product was precipitated with diethyl ether and dried. The coupling step was repeated 3 times to obtain conversion >80%, **Figure S3**. MeO-PEG-Ala-Fmoc product was recrystallised first from DMF by adding diethyl ether, followed by recrystallisation with ethanol. The conversion was determined with H^1-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.

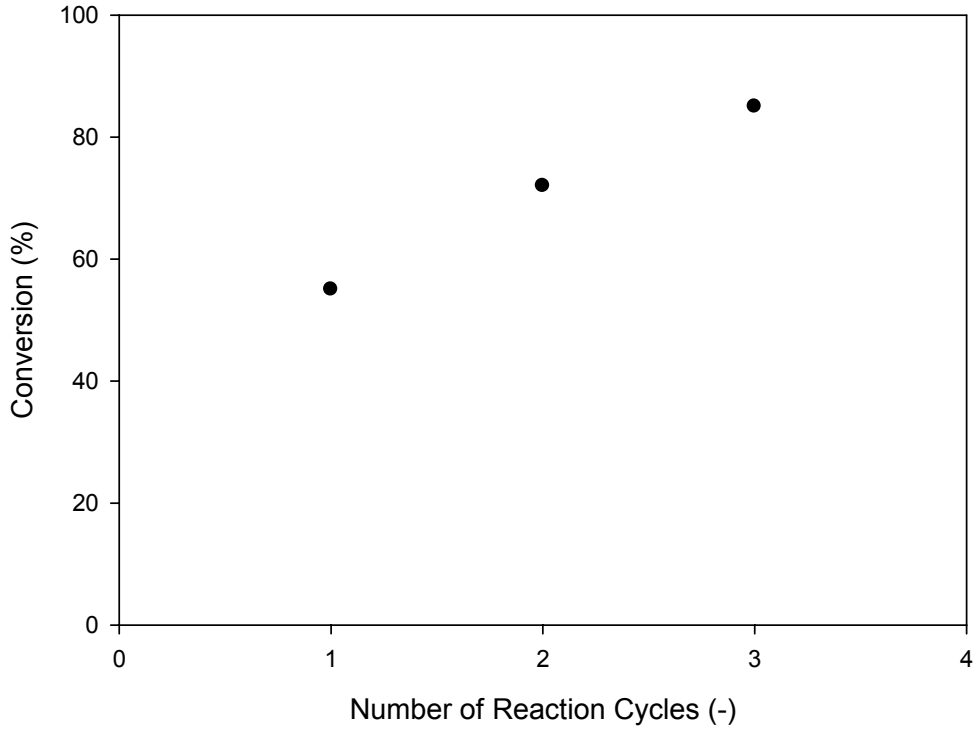

Figure S3: The reaction conversion for Fmoc-Ala attachment onto MeO-PEG as a function of the number of reaction cycles performed. Conversion > 80% is achieved after 3 coupling reaction cycles.

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*. H^1-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.* [S2] was modified by incorporating membrane separation steps. 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, availability in different sizes and shapes, and low cost. In addition it has been shown that the PEG anchoring group has only a minor influence upon the peptide conformation. Another advantage of PEG as anchor is that it is transparent in the visible and the ultraviolet range to 190 nm and solubilises peptides in many different solvents thus allowing for direct CD (Circular Dichroism) analysis of peptide conformation in variety of solvents [S3].

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 pre-activated in DMF for 15 minutes before being added into the PEG solution and mixed vigorously for 2 hours. The product was precipitated with diethyl ether at 4°C for 2 hours 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 H^1-NMR analysis. Conversion was estimated based on 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 pre-dissolved 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 pre-activated for 15 minutes in DMF (10 L per mol MeO-PEG-HMPA) before being mixed with MeO-PEG-HMPA solution for 1 hour. Upon reaction completion the excess reagents were removed by constant volume diafiltration (refer to the main text **Figure 4** 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 H^1-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 pre-swollen in DMF (3 volumes per bed volume) for 30 minutes 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 pre-activated for 15 minutes in DMF (2 volumes per bed volume) before being added to the wet resin. The resulting mixture was shaken vigorously for 1 hour, followed by microfiltration and washes (5 x 2 volumes per bed volume).

Chain assembly with Fmoc-amino acid for SPPS and MEPS. For the MEPS process, Fmoc-amino acid was pre-activated 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 minutes. A separate solution of MeO-PEG-HMPA-*aa*$_{(1)}$-H in DMF was prepared and mixed with the pre-activated solution. The resulting solution was mixed vigorously for 1 hour followed by diafiltration (10 volumes per starting volume). For the SPPS process, Fmoc-amino acid was pre-activated 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 minutes. The solution was added to the swollen Wang resin and the

resulting mixture was shaken vigorously for 1 hour, followed by microfiltration and washes (5 x 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 4**, main text). Deprotection was performed for 30 minutes. Purification after deprotection was performed via diafiltration (12 volumes per starting volume). For the SPPS process, a standard 20% piperidine in DMF solution was pre-mixed and added to the peptide-resin (2 volumes per bed volume). The mixture was shaken vigorously for 30 minutes, followed by microfiltration and washes (6 x 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 re-dissolved into 20 ml per mmol of acidolysis solution for 3 hours. 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 with the solid resin for 3 hours. 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.

Analysis

Ultra-Violet (UV) spectrometry. To verify the presence of the tosylate and phthalimide groups the UV absorption was measured in DCM solution using a Shimadzu UV-2101 PC UV spectrometer, scanned between 200 – 350nm.

Gel Permeation Chromatography (GPC). PEG-peptide losses between processing steps, were measured with a Waters GPC system equipped with both Gilson 132 refractive index (RI) detector and Waters 996 Photodiode Array detector scanned between 250 – 300nm. A Waters Styragel HT2 GPC column was used with N-methyl pyrrolidone (NMP) solvent as mobile phase and operating at a constant flow rate of 0.5 $ml.min^{-1}$ and 100°C. The yield of the MeO-PEG-peptide products was calculated according to **Equation S1**.

$$Yield = \left(\frac{mol \quad of \quad peptide}{mol \quad of \quad amino\text{-}PEG} \right) \times 100 \qquad \textbf{Equation S 1}$$

Nuclear Magnetic Resonance (NMR). The two-dimensional spectrum of MeO-PEG-HMPA and MeO-PEG-HMPA-Tyr-Fmoc were recorded at 298K at 400MHz with a Bruker DRX-400 spectrometer, and analysed with MestRe-C software. The loading of HMPA linker and the first amino acid were estimated by integrating the peaks for aromatic system 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).

Reverse Phase High Performance Liquid-Chromatography (RP-HPLC). The final purity of peptide product, and the level of residual impurities in the post-reaction mixture during diafiltration washes, were measured with an Agilent HPLC system. A

reverse-phase HPLC column (ACE C-18, 250mm×4.6mm) packed with 5μm diameter silica particles with 300Å pore size was operated at 30°C. Water and acetonitrile (AcCN) with 0.1% trifluoroacetic acid (TFA) was used as mobile phase, at 1 $ml.min^{-1}$ flow rate. A ramp from 0% AcCN to 80% AcCN in 30 minutes was followed by 5 minutes at 100% AcCN and 5 minutes at 0% AcCN to wash the HPLC column after each run. UV detection had wavelength set at 210nm.

Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry

The molecular weight of peptide products was measured by Bruker Reflex IV MALDI-TOF mass spectrometer. Infrared ionisation was used with ionisation power set at 20kV. Alpha-cyano-4-hydroxycinnamic acid was used as a matrix in acetoniltrile and ethanol (1:1 ratio) with 0.1% trifluoroacetic acid.

MEPS experimental procedure

The MEPS experimental procedure and set-up is described in **Figure 4** (main text). Both coupling and deprotection steps were performed in the Reaction Vessel at atmospheric pressure. The Circulation Pump recirculated the reaction solution through the membrane cartridge and ensured good liquid mixing throughout. Upon completion of each reaction, the system was pressurised using N_2 to ~7 barg. The resulting solvent flow permeating through the membrane was 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 was applied at each reaction/washing cycle.

To minimize product losses it is desirable that the membrane rejection (**Equation S2**) of the MeO-PEG-peptide is as close to 100% as possible. Throughout the reaction course

the rejection of the MeO-PEG-peptide increased from 99% (for the MeO-PEG-HMPA) to >99.7% after the first and the second amino acid attachment and to ~100% for later attachments. Stable permeation fluxes of 27 – 30 $Lm^{-2}h^{-1}$ at 7 bar operating pressure were achieved during the washes. A higher permeation flux is achievable simply by increasing the pressure applied to the system, which could make the washing step even more time efficient.

$$\text{Rejection of Species i,} \quad R_i\,(\%) = \left(1 - \frac{C_P^i}{C_R^i}\right) \times 100\% \qquad \textbf{Equation S2}$$

where C^i_P is the concentration of solute *i* at the permeate side of the membrane and C^i_R is the concentration of solute *i* at the retentate side of the membrane.

Peptide purity and yield

HPLC analyses of TP-5 produced by both MEPS and SPPS are illustrated in the main text (**Figure 2**). The purity of the MEPS product was estimated as 94%. Purity estimations were based on the HPLC results and obtained as a ratio between 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. Apparently further development and optimisation of the MEPS process is necessary to overcome this obstacle. The MALDI-TOF analysis shown in **Figure 3** (main text) confirmed product molecular weight of MH+ 680. The two impurities (at

10.0 minutes and 10.4 minutes) were identified as peptides resulting from the deletion of Lys, MH+ 550 and Asp, MH+ 564. The TP-5 produced by the SPPS method under the same conditions of 2 equivalents reagents per 1 equivalent peptide and single reaction cycle, was only 77% pure. The main impurity from SPPS was identified as deletion of Arg, MH+ 524, as shown in **Figure 3**.

A 0.9 mmol (MeO-PEG-NH_2 (5)) batch of TP-5 was produced which yielded ~0.6 g of product respectively as estimated from the HPLC analysis of the crude peptide and HPLC calibration curve prepared from TP5 standard. The overall yield of TP-5 was estimated as 92%, with respect to the starting MeO-PEG-NH_2 material (**Equation S1**). 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 non-converted MeO-PEG will be carried throughout the whole process as inert material that increases solution viscosity and reduces the filtration efficiency. Initially, to synthesize MeO-PEG-NH_2 we used the method proposed by *Pillai et al.* [S1] (**Figure S1**) who reported a conversion of 80%. This method involves numerous steps and reagents and was found to be long and highly sensitive to the moisture content of PEG with not easily reproducible conversion. Hence an alternative method for MeO-PEG-NH_2 production was developed based on a simpler reaction procedure, attachment of Fmoc-Ala to MeO-PEG (**Figure S2**). The latter method is less sensitive to the moisture content of PEG, with reproducible conversion >80% after 3 reaction cycles and utilises only reagents used in peptide synthesis. This improved the efficiency of the MEPS process.

Table S1: List of reagents and protecting groups used in the MEPS/SPPS peptide synthesis

Compound symbol	Compound name	Nature
Boc	t-Butoxycarbonyl	Protecting group
DCM	dichloromethane	Solvent
DIC	N,N'-diisopropylcarbodiimide	Activator
DIPEA	diisoproyl ethyl amine	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-t-butoxycarbonyl-L-arginine	Amino acid
Fmoc-Asp (O^tBu)-OH	N-α-Fmoc-L-aspartic acid β-t.-butyl ester	Amino acid
Fmoc-Lys (Boc)-OH	N-α-Fmoc-N-ε-t.-Boc-L-lysine	Amino acid
Fmoc-Tyr (^{t}Bu)-OH	N-α-Fmoc-O-t.-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

References

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S3 C. Toniolo, G. M. Bonora, M. Mutter, *J. Am. Chem. Soc.,* 1979, **101**, 450-454.