

Short oligoalanine helical peptides for supramolecular nanopore assembly and protein cytosolic delivery

Marta Pazo, Giulia Salluce, Irene Lostalé-Seijo, Marisa Juanes, Francisco Gonzalez, Rebeca Garcia-Fandiño and Javier Montenegro

Supporting information

Copyright information:

© 2021 The Author(s). Published by the Royal Society of Chemistry

Short Oligoalanine Helical Peptides for Supramolecular Nanopore Assembly and Protein Cytosolic Delivery

Marta Pazo, Giulia Salluce, Irene Lostalé-Seijo, Marisa Juanes, Francisco Gonzalez, Rebeca Garcia-Fandiño and Javier Montenegro*

e-mail to: javier.montenegro@usc.es

Supporting Information

Table of contents

1.	Supporting Figures	2
2.	Experimental Procedures	17
2.1.	Materials	17
2.2.	Abbreviations	18
2.3.	General protocol for the SPPS	18
2.3.1.	Synthesis of peptide MP1	19
2.3.2.	Synthesis of peptide CF-MP1	19
2.3.3.	Synthesis of peptide TM-MP1	19
2.3.4.	Synthesis of peptide MP2	19
2.3.5.	Synthesis of peptide P3	19
2.3.6.	Synthesis of peptide Arg8	19
2.3.7.	Synthesis of peptide GALA	20
2.3.8.	Synthesis of peptide L17E	20
2.3.9.	Synthesis of Pep-1	20
2.3.10.	Synthesis of dfTAT	20
2.4.	Liposome preparation	20
2.5.	ANTS/DPX assay	21
2.6.	Dextran release in vesicles	21
2.7.	Circular Dichroism	22
2.8.	DLS experiments	22
2.9.	FRET and fluorescence experiments	22
2.10.	Computational methods	23
2.11.	Planar lipid bilayer recordings	23
2.12.	Cell culture experiments	24
2.12.1.	Cell lines and culture.....	24
2.12.2.	Cellular uptake and endosomal release of dextran.....	24
2.12.3.	Cell viability assay (MTT)	25
2.12.4.	Saporin experiments.....	25
2.12.5.	Cre recombinase expression and purification	25
2.12.6.	Cre recombinase delivery experiments	25
2.12.7.	Antibody delivery in cell culture.....	26
2.12.8.	CF-labelling and delivery of IgG	26
2.12.9.	Purification and delivery of GST-NLS-GFP.....	26
2.13.	Ex vivo cornea experiments	27
2.13.1.	Murine corneas culture.....	27
2.13.2.	Antibody delivery in cornea.....	27
2.13.3.	Live/Dead Staining.....	27
2.14.	Sources for Figure 6 table	27
3.	Supporting References	28

1. Supporting Figures

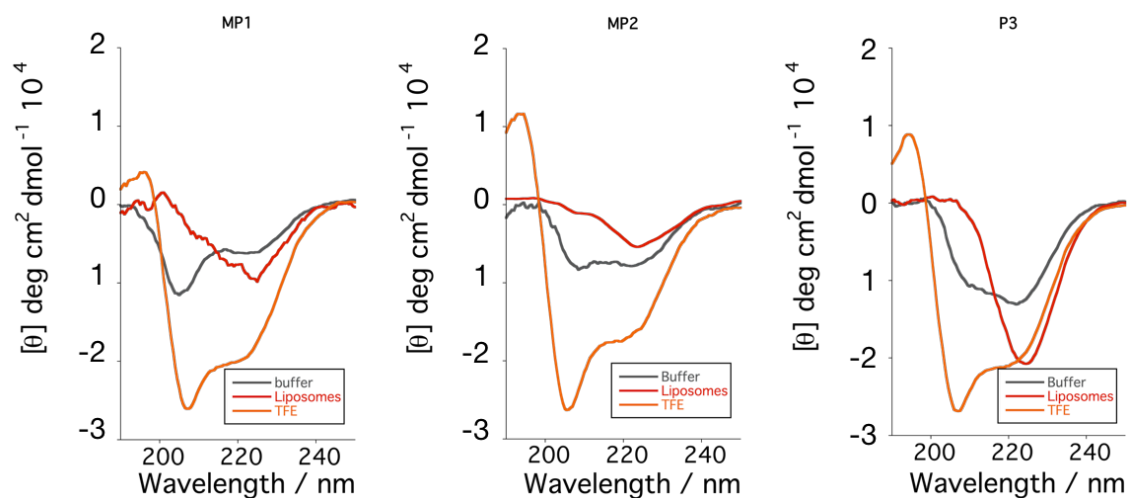


Figure S1. Circular dichroism spectra of MP1, MP2 and P3 in HKR buffer (gray), Liposomes (red) (L/P ratio: 12) and TFE (orange). Measurements were done at 40 °C. See also Fig. 1d.

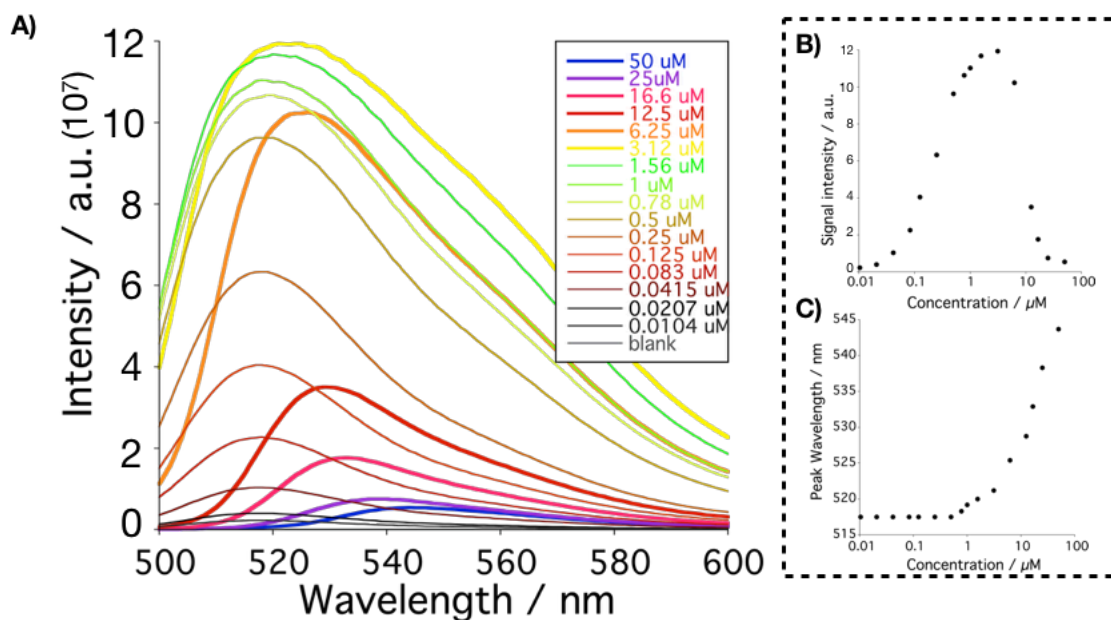


Figure S2. CF-MP1 aggregates in solution. A) Emission spectra of CF-MP1 ($\lambda_{\text{ex}} = 494 \text{ nm}$) in aqueous solution at different concentrations. B) Maximum fluorescence intensity at different concentrations C) Wavelength of the peak of maximum fluorescence at different concentrations.

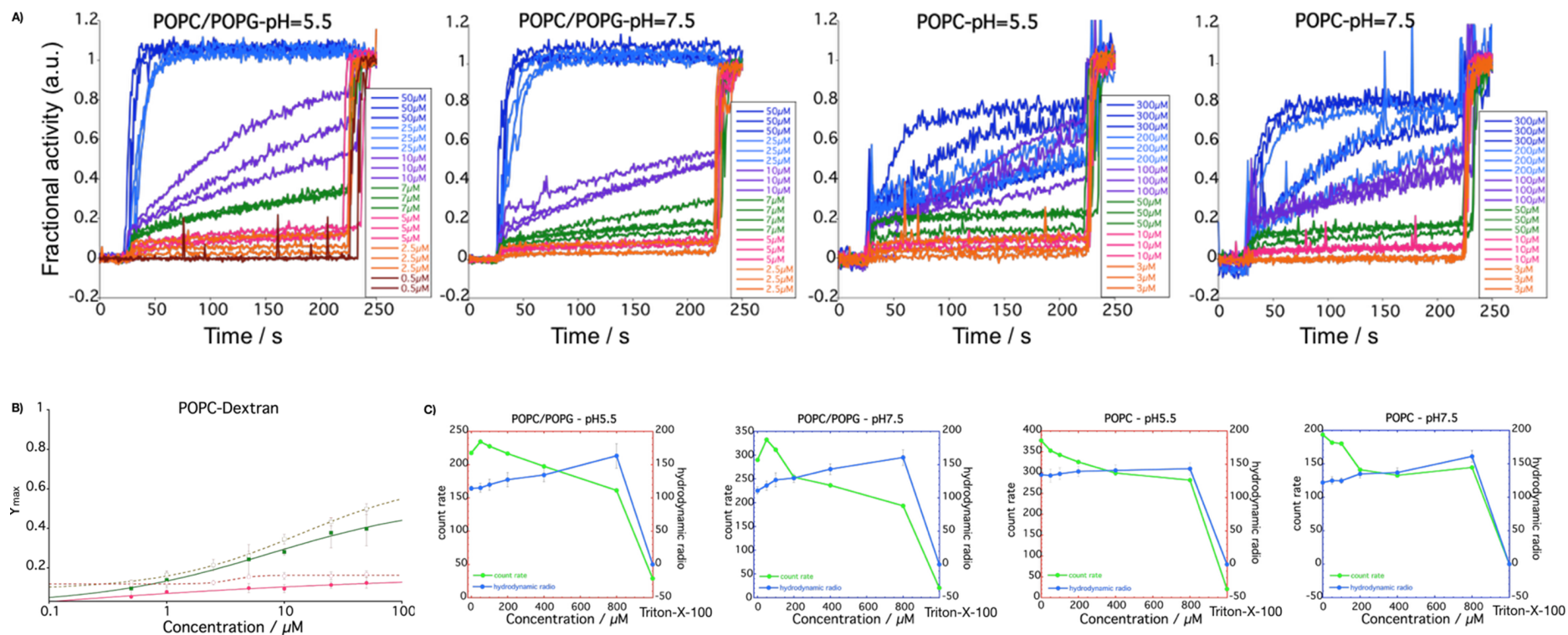


Figure S3. Vesicle release experiments. A) Kinetic fluorescent traces of LUVs_CANTS/DPX after addition of MP1 (at $t = 25$ s). Triton X-100 was added at 225 s for complete vesicle lysis and normalization. B) Dose response curves for dextran release in neutral POPC vesicles (LUVs_CDextran) at different MP1 concentrations. The 10 kDa FITC-Dex (green) and 40 kDa TM-Dex (pink) are shown with empty squares (pH = 7.5) and filled circles (pH = 5.5) respectively. C) DLS measurements of vesicles in the presence of MP1. Representation of photon count rate (an indicator of measurement quality) and size (hydrodynamic radius, indicative of vesicle size and stability). Error bars indicate the standard deviation of three replicates calculated by equation S5 for DLS experiments. In all cases the buffer was MES 10 mM with NaCl 150 mM. Experiments were done with neutral POPC or anionic POPC/POPG vesicles at either pH 7.5 or pH 5.5 as indicated at the top of the charts.

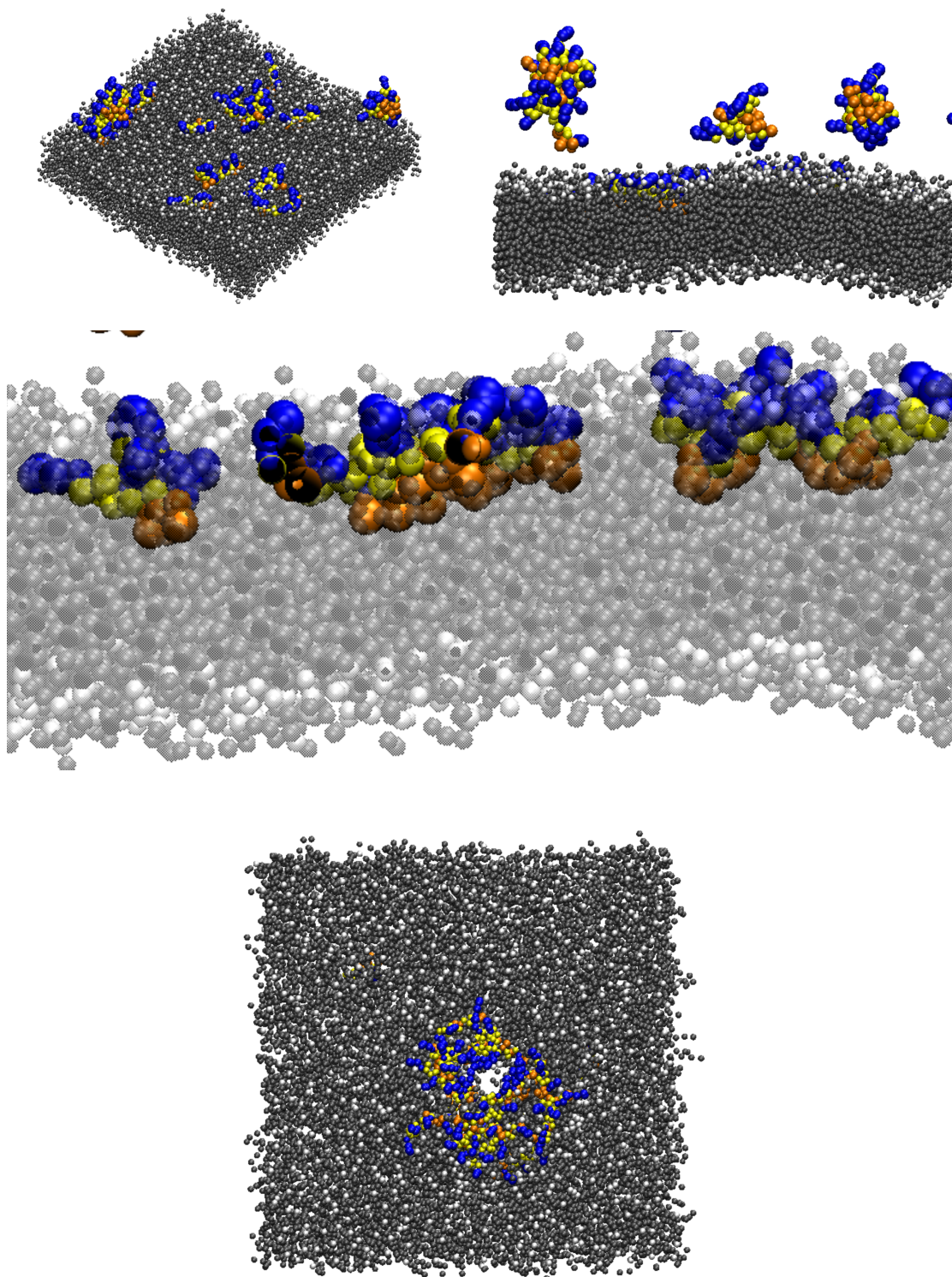


Figure S4. Molecular dynamics simulation. Top: Snapshot at $t = 70$ ns of the CG-MD simulation of 31 units of peptide **MP1** interacting with a membrane composed by DPPC:DPPE (9:1) and DPPC:DPPE:DPPS (3:5:2), in absence of electric field. Middle: Detail of the insertion of the peptides in the membrane by anchoring and orienting the Leu (orange) enriched hydrophobic N-terminus of peptide **MP1** towards the hydrophobic core of the lipid bilayer, and the arginine (blue) cationic residues exposed to the solvent. Down: Detail of the top view of the ensemble of **MP1** interacting with the membrane, under an external electric field of 0.03 V/nm applied across the membrane ($t = 300$ ns, from Figure 5). An initial poration of the membrane can be inferred.

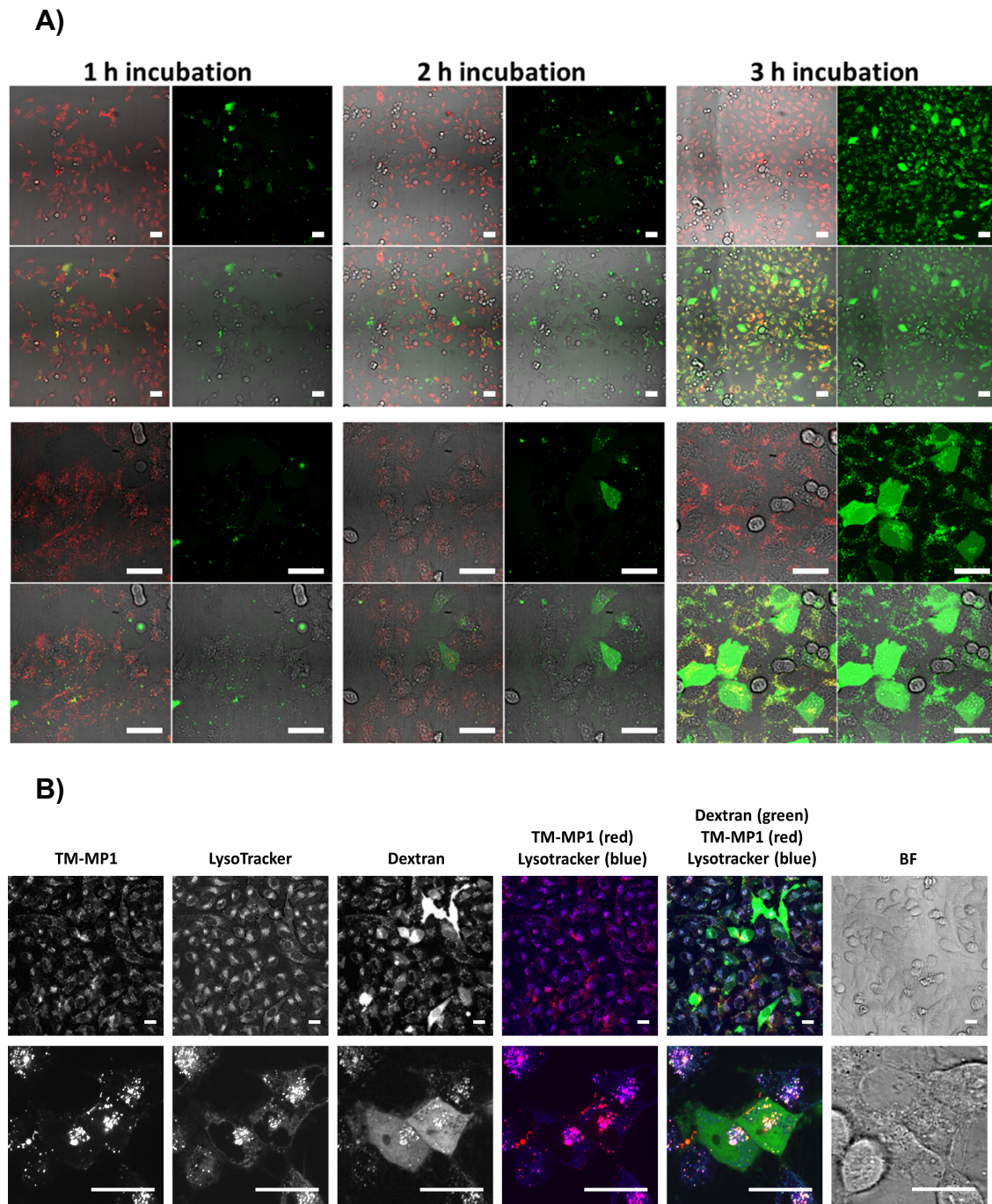


Figure S5. Time lapse of dextran cytosolic release in HeLa cells. A) HeLa cells were incubated with 0.25 mg/mL of Alexa₄₈₈-Dextran (10 kDa, green) in the presence of 5 μ M of **TM-MP1** (red) for 1 h in DMEM without FBS. Cells were washed and incubated in DMEM with FBS for 1 hour, 2 hours or 3 hours before imaging. Top rows: 20x magnification; bottom rows: 60x magnification. Each set of images shows: merge of brightfield and **TM-MP1** (red; upper left), dextran fluorescence (green; upper right), merge of brightfield and dextran (lower right), merge of **TM-MP1**, dextran, and brightfield (lower left). Scale bars = 50 μ m. B) HeLa cells were incubated with 0.25 mg/mL of FITC-Dextran (10 kDa, green), in the presence of 5 μ M **TM-MP1** (red) for 1 h in DMEM without FBS. Cells were washed and incubated in DMEM with FBS for 3 h and then stained with LysoTracker Deep Red (100 nM, 30 min, shown in blue), washed and observed at the microscope. Scale bars = 25 μ m.

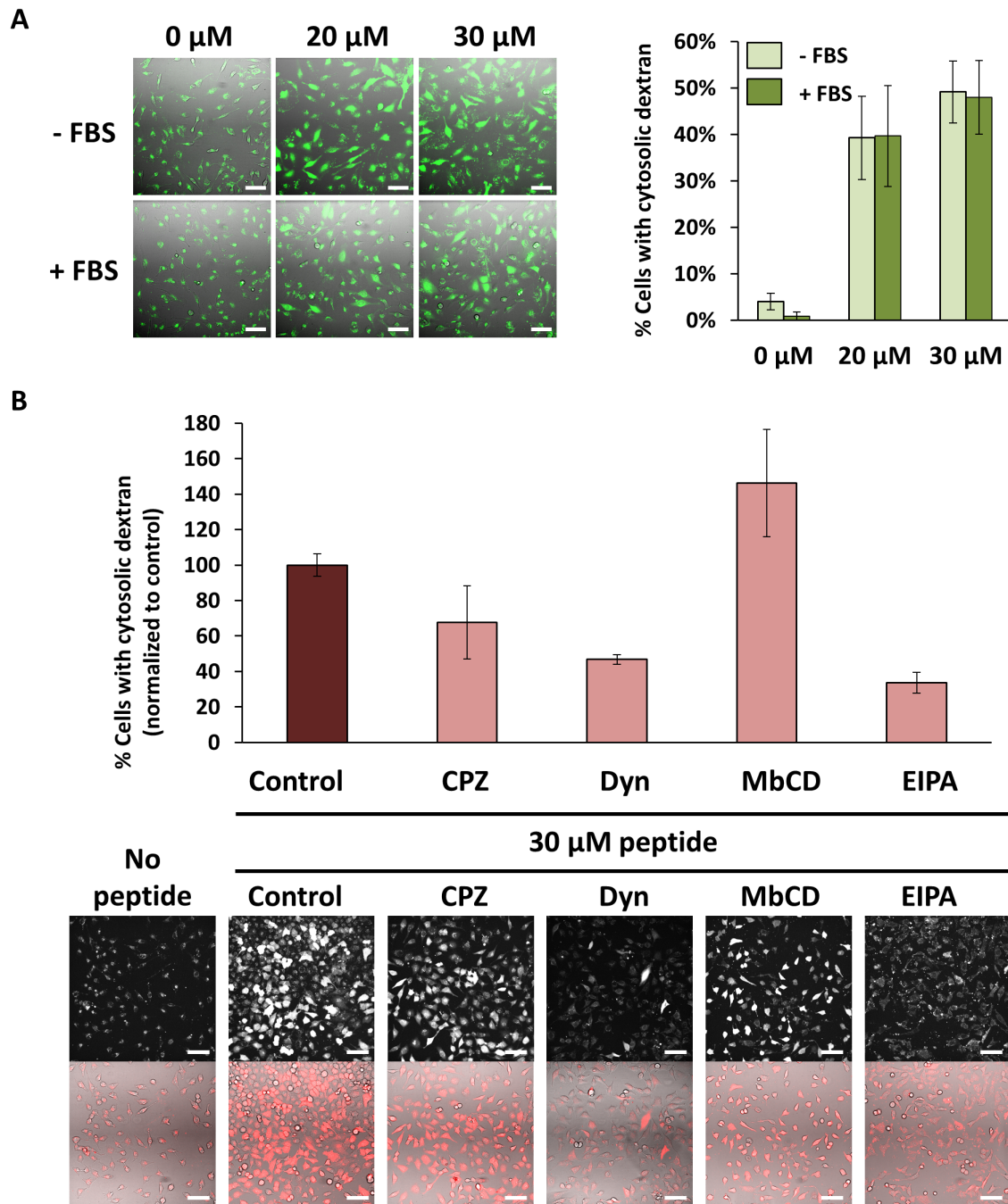


Figure S6. Dextran release in the presence of serum or endocytosis inhibitors. A) HeLa cells were incubated for 1 h with a solution of 0.25 mg/mL 10 kDa Alexa₄₈₈-Dextran (green) and the indicated concentrations of **MP1** in DMEM stock (top row) or in DMEM supplemented with 10% FBS (bottom row). Cells were then washed and incubated for 3 hours with DMEM supplemented with 10% FBS before confocal imaging. Cells with diffuse signal were counted and expressed as percentage. Graph shows mean of three experiments \pm SD. B) HeLa cells were incubated with 0.25 mg/mL 10 kDa TM-labelled dextran and 30 μ M peptide **MP1** in the presence of the indicated inhibitors for 1 h. After incubation, the number of cells with cytosolic dextran was counted and normalized to the control with 30 μ M peptide in the absence of inhibitors (100%). Data is presented as mean of three independent experiments \pm SD. Representative micrographs for each condition are shown at the bottom of the image (top row: dextran fluorescence; bottom row: merge of dextran fluorescence (red) with brightfield). Scale bars = 100 μ m.

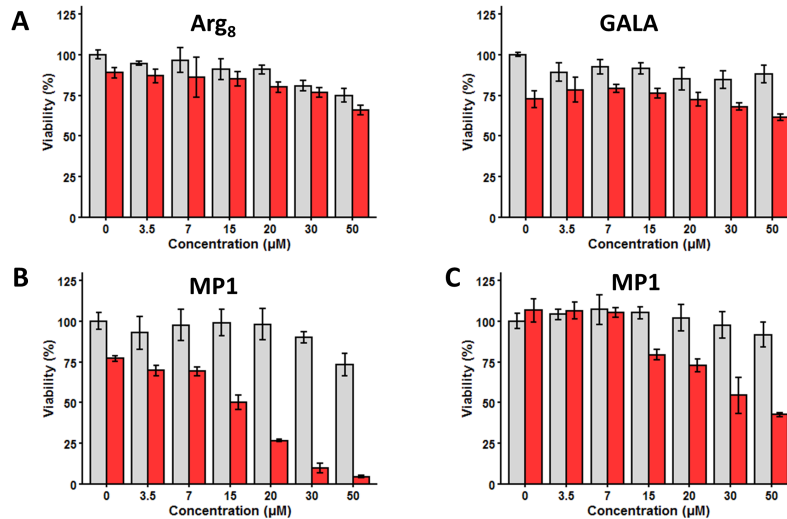


Figure S7. Functional Protein Cytosolic Delivery. A) HeLa cells were incubated with 0 (grey bars) or 10 µg/mL (red bars) saporin in the presence of increasing concentrations of peptides **Arg8** or **GALA** for 1 h; medium was then replaced and cells further incubated for 6 h before measuring viability by MTT assay. B, C) Analogous experiment in HeLa (B) and A549 (C) cells by incubation with **MP1** (1 h) and further incubation for 24 h before viability quantification by MTT assay.

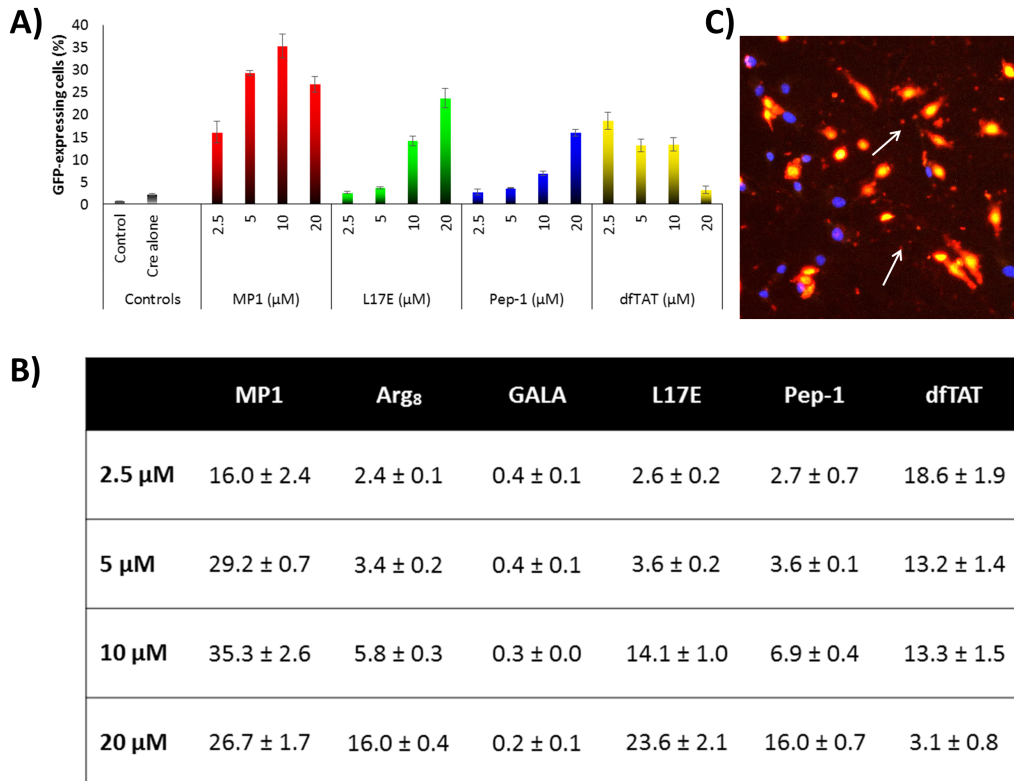


Figure S8. Cre recombinase delivery efficiency by different peptides. A) and B) Cre-reporter HeLa cells were treated with 1 µM Cre recombinase and 2.5, 5, 10, 20 µM of the indicated peptides for 4 h, and the number of cells that switched from red to green (GFP) fluorescence due to the recombinase activity quantified by flow cytometry 3 days after incubation. The table indicates the percent of GFP-expressing cells ± SD (n = 3) for each peptide and concentration. C) **TM-MP1** and Cre form aggregates. HeLa-Cre reporter cells were incubated with 1 µM Cre recombinase and 20 µM TAMRA-labelled **MP1** (TM-MP1) for 4 h, and cells imaged 3 days later. Both TAMRA (peptide) and dsRED (expressed by cells) are shown in red, GFP in green and Hoechst-stained nuclei in blue. Arrows indicate fluorescent peptide aggregates as detected by the TAMRA fluorescence, which may be responsible of the lower uptake at high concentrations.

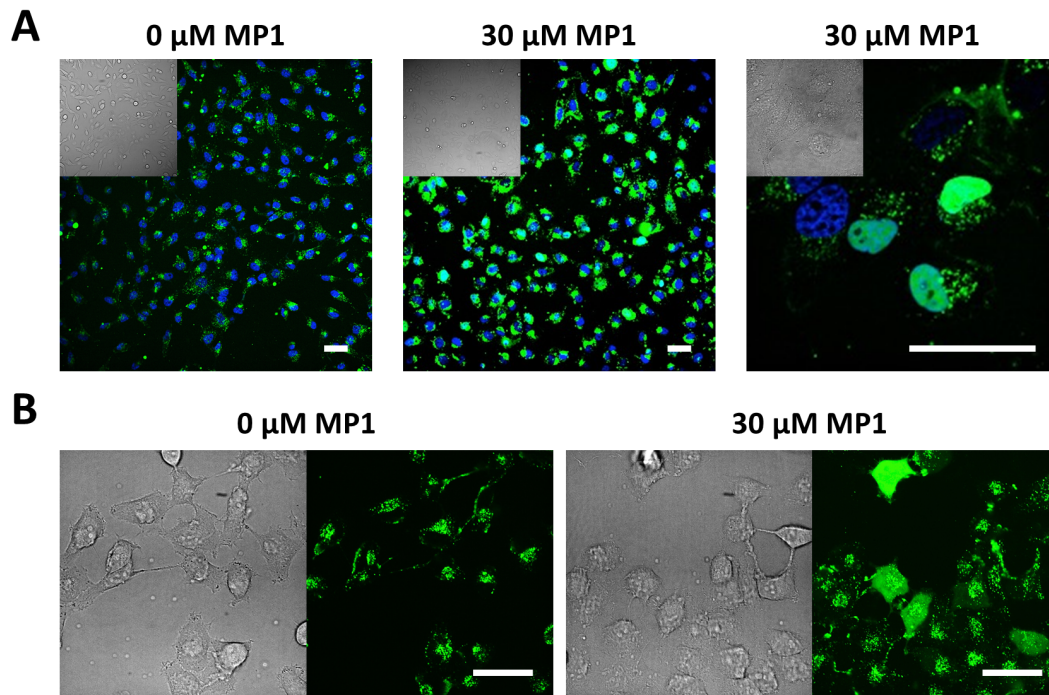
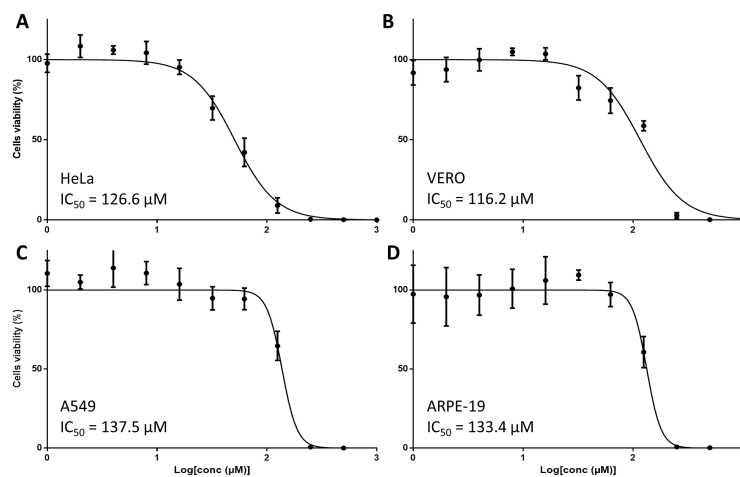


Figure S9. Delivery of GST-NLS-GFP and IgG-CF into HeLa cells. A) HeLa cells were incubated in DMEM with GST-NLS-GFP (10 μM ; shown in green) in the absence or presence of 30 μM MP1 for 1 h, washed and further incubated for 90 min in DMEM supplemented with 10% FBS before confocal imaging. Nuclei were stained with Hoechst (blue). Around 15% of the cells showed nuclear distribution of GST-NLS-GFP. B) HeLa cells were incubated with IgG-CF (1 mg/mL, green) in the presence or absence of 30 μM MP1 for 1 h, in the same conditions as in A. Scale bars = 50 μm .



IC_{50} MP1	HeLa	VERO	A549	ARPE-19
	126.6 μM	116.2 μM	137.5 μM	133.4 μM

Figure S10 MTT viability assays. Viability was determined in HeLa (A), Vero (B), A549 (C) and ARPE-19 (D) cells. Cells were incubated with different concentrations of MP1 (0, 1, 2, 4, 8, 16, 32, 62.5, 125, 250, 500, 1000 μM) in DMEM for 1 h, then the solutions were removed and replaced with complete medium. After 24 hours, cells were incubated for 3 hours with medium supplemented with 0.5 mg/mL MTT tetrazolium salt, and the medium was removed before solubilizing the formazan salt with DMSO. Cells viability was then quantified by absorbance. Data was normalized to the untreated control (100 %). Data is shown as mean of five replicates \pm SD.

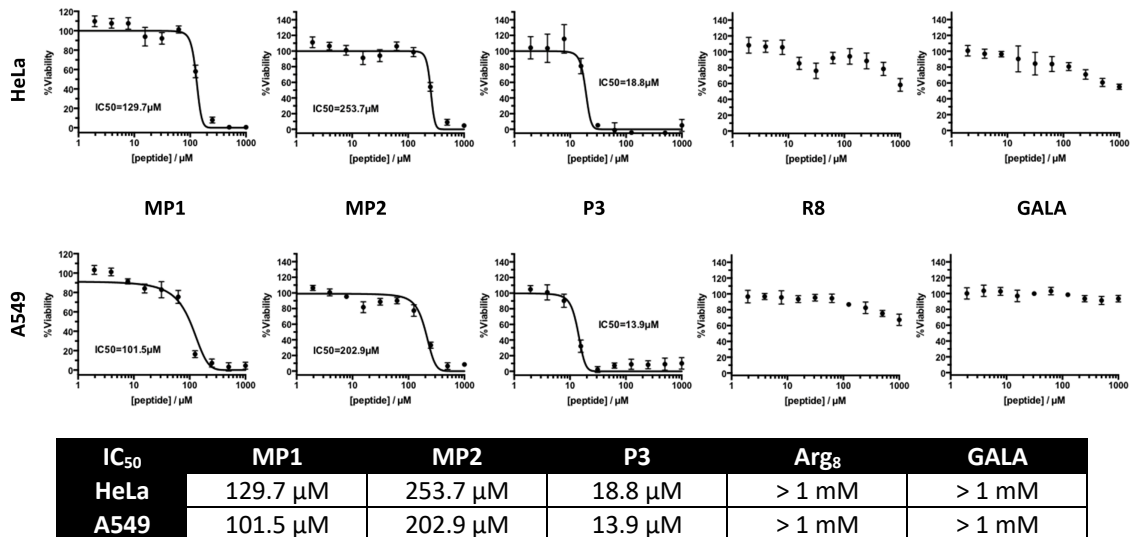


Figure S11. MTT viability assays at 1 h of incubation. HeLa (top row) or A549 (bottom row) were incubated with increasing concentrations of the indicated peptides in DMEM for 1 h, and then the solutions were removed and replaced by medium supplemented with 0.5 mg/mL MTT, incubated for 1 h, and, after medium removal, formazan salt was solubilized with DMSO and viability measured by absorbance. Data was normalized to untreated control (100%) and represented as mean \pm SD ($n = 5$).

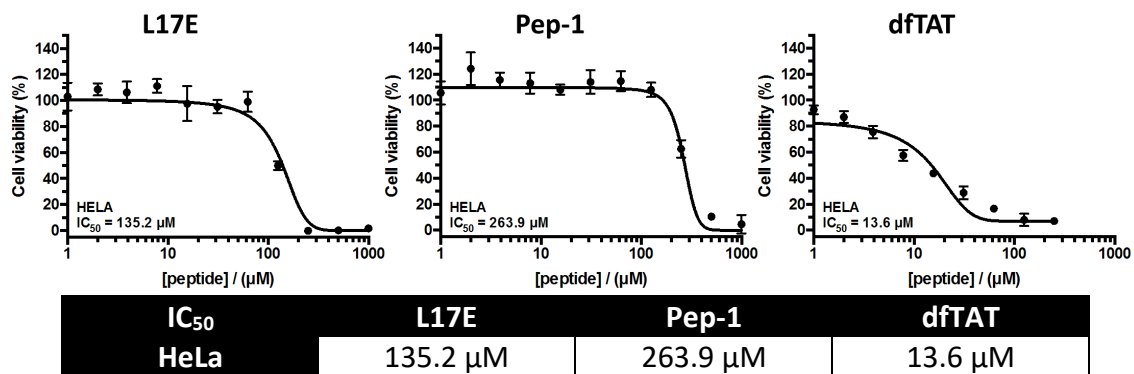


Figure S12. MTT assay of control protein delivery peptides. Viability was determined in HeLa cells, after 1 h of incubation with different concentrations of the indicated peptides in DMEM, followed by 1 h incubation with medium supplemented with 0.5 mg/mL MTT tetrazolium salt. Formazan salt was solubilized with DMSO after medium removal and the absorbance at 570 nm measured. Data were normalized to the untreated control (100% viability) and shown as the mean of five replicates \pm SD. As indicated in Fig. S11, MP1 $IC_{50} = 129.7 \mu\text{M}$.

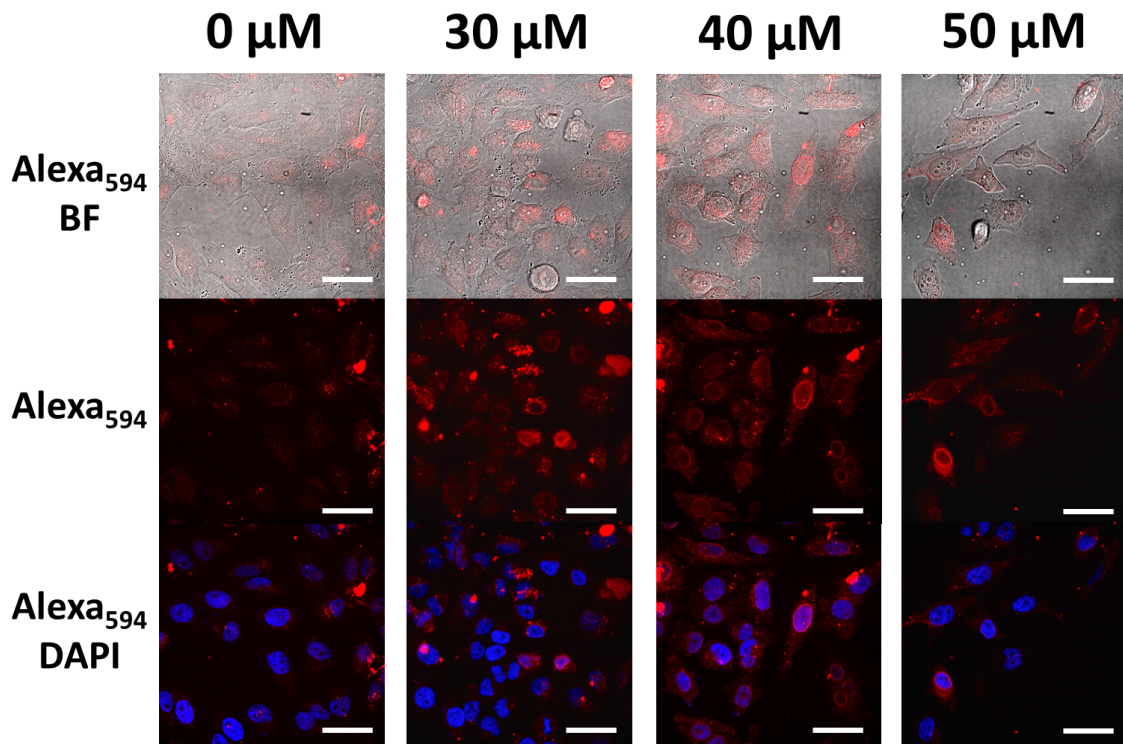


Figure S13. Cytosolic delivery of antibodies in HeLa cells. HeLa cells were incubated with 0.3 mg/mL of Mab414 and increasing amounts of MP1 for 1 h. Cells were thoroughly washed and incubated for 3 h before fixation and immunofluorescence with Alexa Fluor 594 conjugated secondary antibody (Alexa₅₉₄, red). Nuclei were stained with DAPI (blue). Scale bars = 50 μ m.

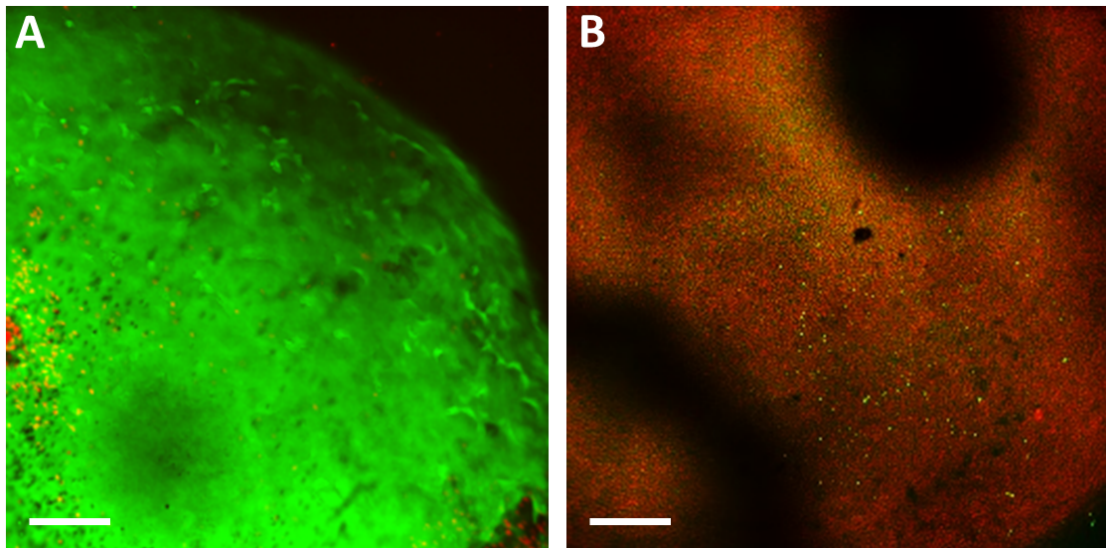


Figure S14. Delivery of antibodies in *ex vivo* corneas. Maximum projections of live/dead staining of murine corneas with propidium iodide (PI)/fluorescein diacetate (FDA). FDA (green) stains live cells' cytosol and PI (red) stains dead cells' nuclei. A) live cornea control. B) dead control. Live control was incubated in Ames' for 1 h, while dead control was incubated with 0.5% Triton X-100 for 1 h. Both were further incubated with Ames's + 10% FBS for 2 h before staining. Scale bars = 200 μ m.

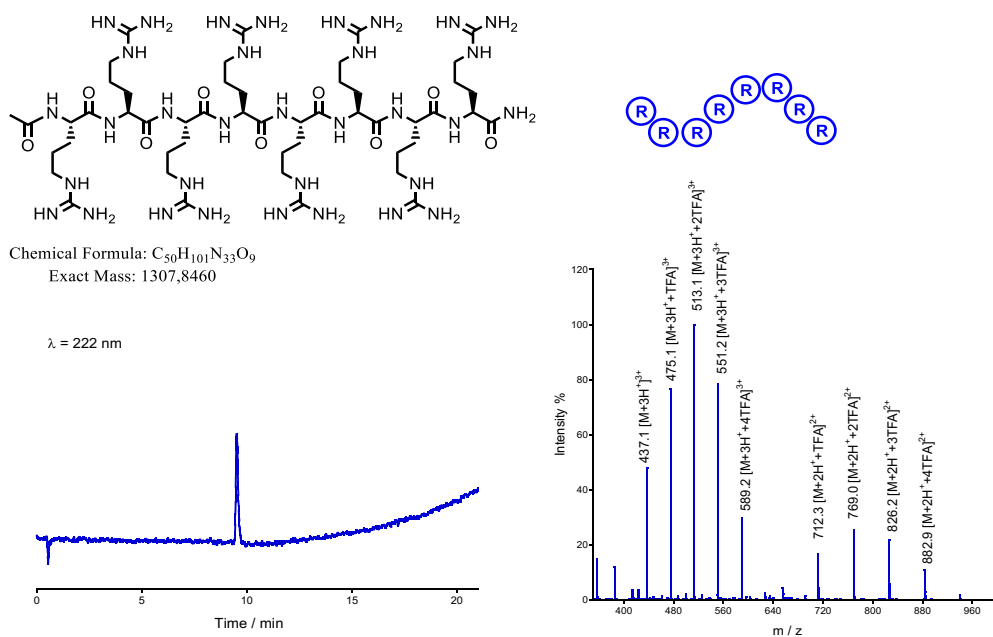


Figure S19. Arg8 characterization. RP-HPLC [Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*_t 9.5 min) absorbance at 222 nm and ESI-MS for Peptide Arg8.

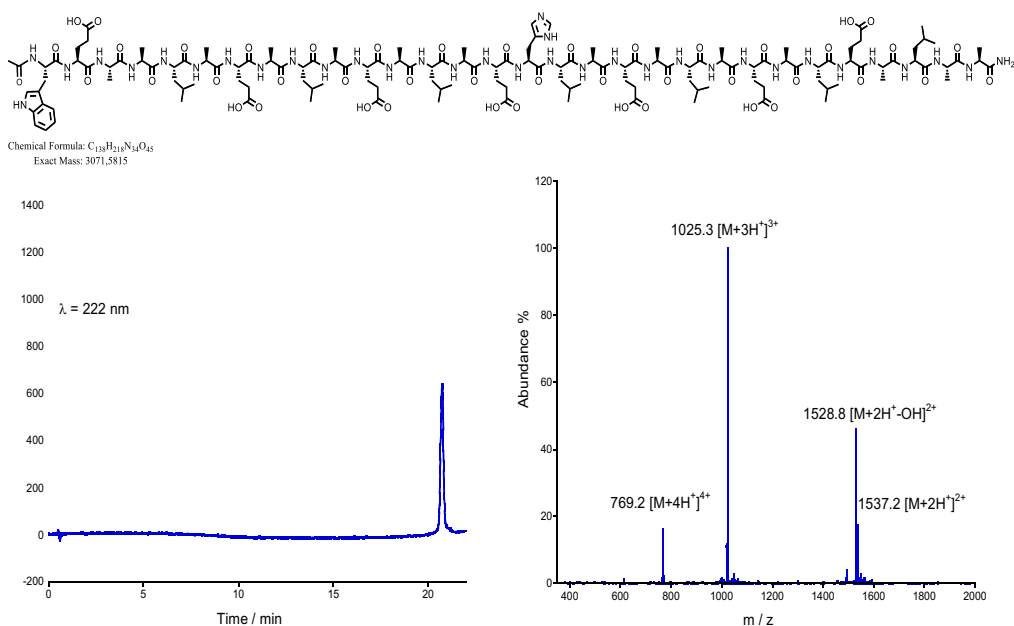


Figure S20. GALA characterization. RP-HPLC [Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→21 min)] (*R*_t 20.6 min) absorbance at 222 nm and ESI-MS for Peptide GALA.

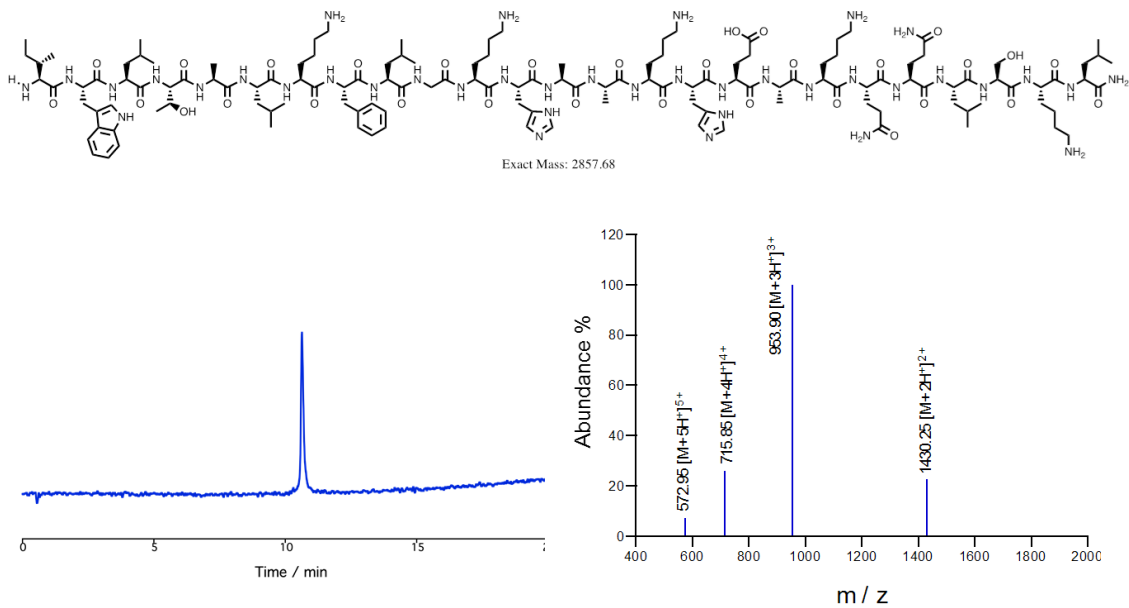


Figure S21. L17E characterization. RP-HPLC [Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*_t 10.6 min) absorbance at 222 nm and ESI-MS for Peptide **L17E**.

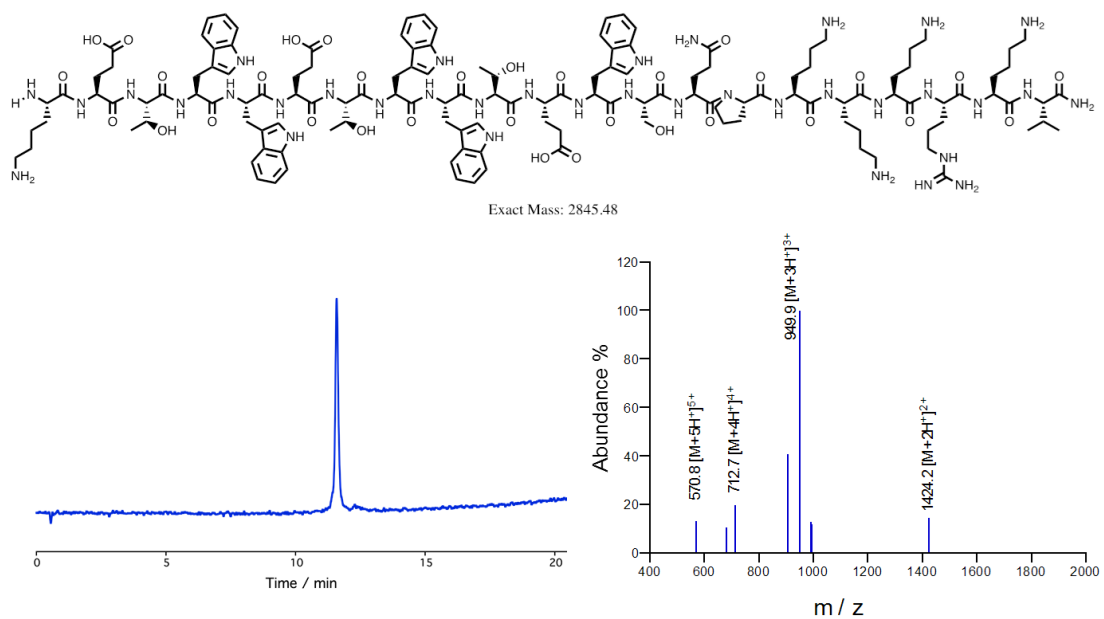


Figure S22. Pep-1 characterization. RP-HPLC [Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R*_t 11.6 min) absorbance at 222 nm and ESI-MS for Peptide **Pep-1**.

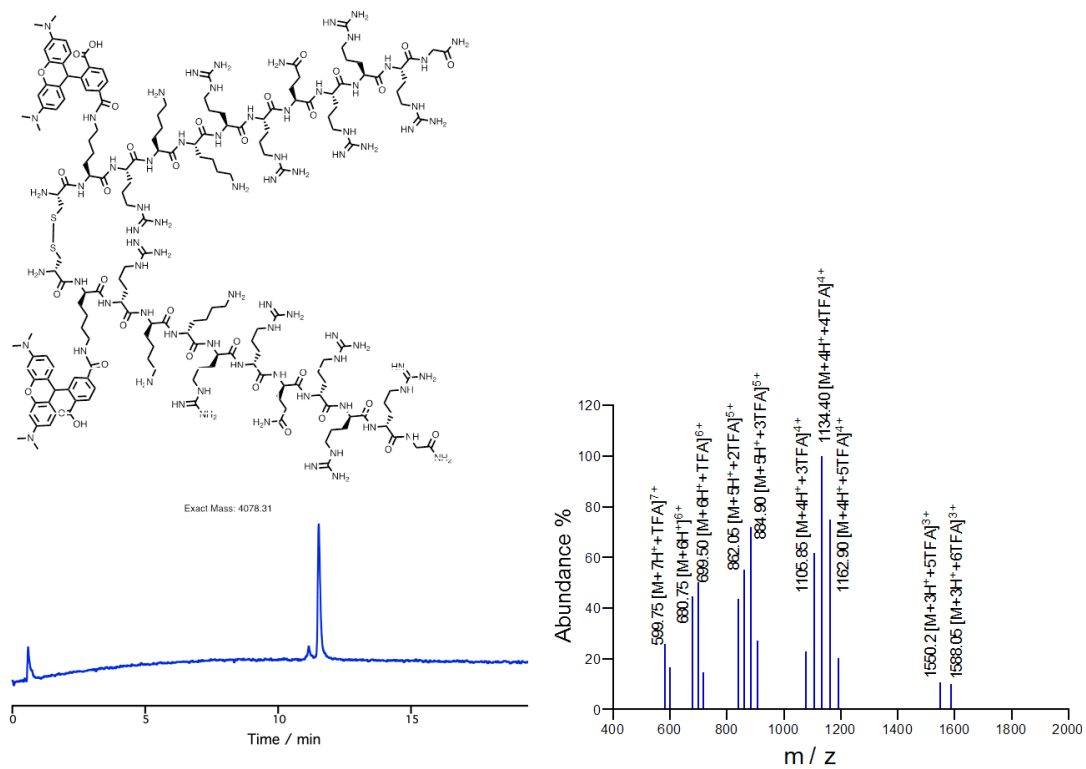


Figure S23. dFTAT characterization. RP-HPLC [Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)] (*R_t* 11.5 min) absorbance at 222 nm and ESI-MS for dFTAT.

Video S1. Detail of a CG-MD simulations trajectory (5 ns in total) highlighting the initial interaction of **MP1** with a lipid bilayer composed by DPPC:DPPE (9:1) and DPPC:DPPE:DPPS (3:5:2). As it can be noticed, although the preliminary interaction of **MP1** with the membrane is led by electrostatic interactions between the Arg residues and the polar heads of the lipids, the peptide quickly orients the Leu enriched hydrophobic N-terminus of **MP1** towards the hydrophobic core of the lipid bilayer, and the arginine cationic residues remain exposed to the solvent. CG residues are represented in yellow (Ala), orange (Leu) and blue (Arg).

2. Experimental Procedures

2.1. Materials

Commercially available Rink Amide-resin ChemMatrix, Fmoc-*L*-Ala-OH, Fmoc-*L*-Leu-OH, Fmoc-*L*-Arg(pbf)-OH, triisopropylsilane (TIS), Diisopropylethyl amine (DIEA), diisopropylcarbodiimide (DIC), and 5-(*N*-Ethyl-*N*-isopropyl)amiloride (EIPA) were obtained from Sigma-Aldrich. Trifluoroethanol, 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) and chlorpromazine were purchased from TCI. Egg yolk L- α -phosphatidylcholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine in chloroform (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol sodium salt in chloroform (POPG), 16:0-12:0 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-phosphoethanolamine (NBD-PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (PE-NBD) and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) were purchased from Avanti Polar Lipids. Ethyl(hydroxyimino)cynoacetate (Oxyma), methyl- β -cyclodextrin, and 5-carboxytetramethyl rhodamine (TAMRA) were available from Carbosynth. *N*-HATU was provided by Glentham life sciences. *N*-HBTU was obtained from Iris. Peptide synthesis grade *N,N*-dimethylformamide was purchased from Scharlau. Dynasore was from EMD Millipore Corporation. All the other solvents were HPLC grade, purchased from Sigma-Aldrich® or Fisher Scientific®, and used without further purification.

Dulbecco's Modified Eagle's Medium (4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate) was obtained from Gibco. Puromycin was purchased from Enzo Life Sciences. Ames' medium, Alexa Fluor 488 – Dextran (10 kDa), Tetramethylrhodamine – Dextran (10 kDa), fluorescein – Dextran (70 kDa), and LysoTracker Deep Red were acquired from Thermo Fisher. FITC – Dextran (40 kDa), Saporin, Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate), human IgG (#I4506) and anti-nuclear pore complex proteins antibody Mab414 (Mouse monoclonal, clone 414, #N8786) were purchased from Sigma-Aldrich.

A microwave assisted peptide synthesiser (Liberty Lite, CEM) was used to prepare the peptide according to standard methods developed by the manufacturers involving diisopropylcarbodiimide (DIC) 0.5 M in DMF as activator and ethyl(hydroxyimino)cynoacetate (Oxyma) 1 M in DMF as activator base. High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) analyses were carried out on Agilent Technologies 1260 Infinity II associated with a 6120 Quadrupole LC-MS using an Agilent SB-C18 column or on DIONEX Ultimate 3000 U-HPLC⁺ (Thermo Scientific) with an Acclaim RSLC 120-C18 column with *Solvent A:Solvent B* gradients between 5:95 (*Solvent A*: H₂O with 0.1% TFA; *Solvent B*: CH₃CN with 0.1% TFA). High-performance liquid chromatography (HPLC) semi-preparative purification was carried out on Jasco LC-4000 with an Agilent Eclipse XDB-C18 column.

Accurate mass determinations (HR-MS) using ESI-MS were performed on a Bruker MicroTof mass spectrometer.

Circular Dichroism (CD) measurements were performed with a Jasco J-1100 CD Spectrometer equipped with a Jasco MCB-100 Mini Circulation Bath for temperature control.

Vesicles were prepared with a Mini-Extruder from Avanti Polar Lipids (pore size 100 nm). Sample incubation was performed in 500 μ L eppendorf vials. Fluorescence measurements were performed with a FluoroMax-2 spectrofluorometer (Jobin-Yvon Spex) equipped with a stirrer and a temperature controller.

DLS measurements were performed in a Malvern Zetasizer Nano ZSP using standard disposable cuvettes.

For the acquisition of cell microscopy images a Nikon Eclipse Ti-E epifluorescence microscope with an Andor Zyla 4.2 digital camera, or a Dragonfly confocal spinning-disk on a Nikon Eclipse Ti-E equipped with a Andor Zyla 4.2 PLUS sCMOS digital camera were used. Images were processed with FIJI.^{S1} 3D images were reconstructed from the different individual confocal planes with Imaris 9.0.0 software (Oxford instruments).

A Tecan Infinite F200Pro microplate reader was used to directly measure in Costar cell culture 96-well plates UV-Vis absorbance for the MTT viability assays. Flow cytometry was performed on a Guava easyCyte™ cytometer and data analysed with InCyte software included in GuavaSoft 3.2 (Millipore).

2.2. Abbreviations

Aa: Amino acid; Arg: Arginine; BSA: bovine serum albumin; Calcd: Calculated; CF: Carboxyfluorescein; CF-MP1: oligoalanine MP1 labelled with carboxyfluorescein at *N*-terminus; CPZ: Chlorpromazine; DCM: Dichloromethane; Dex: dextran; DIC: diisopropylcarbodiimide; DIEA: *N,N*-Diisopropylethylamine; DMF: *N,N*-Dimethylformamide; DMEM: Dulbecco's Modified Eagle's Medium; DPPC: dipalmitoyl phosphatidylcholine; DPhPC: 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; DPPE: dipalmitoyl phosphatidylethanolamine; DPPS: dipalmitoyl phosphatidylserine; Dyn: Dynasore; EGFP: enhanced green fluorescent protein; EIPA: 5-(*N*-Ethyl-*N*-isopropyl)amiloride; FDA: fluorescein diacetate; GST: glutathione-S-transferase; HFIP: 1,1,1,3,3,3-Hexafluoro-2-propanol; HRMS (ESI): High resolution mass spectrometry (electrospray ionization); IPTG: Isopropyl β -D-1-thiogalactopyranoside; LB: Lysogeny broth; Leu: Leucine; Lys: Lysine; LUV: large unilamellar vesicles; MbCD: methyl- β -cyclodextrin; MES: 4-Morpholineethanesulfonic acid; Leu: Leucine; Lys: Lysine; MP1: minimal peptide 1; MP2: peptide 2 with aligned leucines; MOPS: 3-(*N*-Morpholino)propanesulfonic acid; Mtt: 4-Methyltrytil; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; NBD-PE: 16:0-12:0 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-phosphoethanolamine; *N*-HATU: *N*-[(Dimethylamino)-1H-1,2,3-triazolo[4,5-*b*]pyridine-1-ylmethylene]-*N*-methylmethanaminium-hexafluorophosphate *N*-oxide; *N*-HBTU: *N*-[(1HBenzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; NLS: Nuclear Localization Signal; P3: longer oligoalanine peptide; PE-NBD: 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); Pbf: 2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl; PBS: Phosphate-Buffered Saline; POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PI: propidium iodide; POPG: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; SPPS: solid phase peptide synthesis; SV40: Simian Virus 40; TAMRA: 5-carboxytetramethyl rhodamine; TFE: Trifluoroethanol; TIS: Triisopropylsilane; TM-MP1: oligoalanine 1 labelled with TAMRA at *N*-terminus; TNBS: 2,4,6-Trinitrobenzenesulfonic acid;

2.3. General protocol for the SPPS

All peptides were synthesized by automated or manual Fmoc solid-phase peptide synthesis^{S2} using Rink Amide ChemMatrix resin (loading 0.5 mmol/g). For manual synthesis, the resin (0.5 mmol) was swelled in DMF (peptide synthesis grade, 2 mL) for 20 min in a peptide synthesis vessel prior synthesis. Coupling cycle consisted of the removal of Fmoc protecting group with a solution of piperidine in DMF (20%, 2 mL) for 10 min and then the mixture was filtered and the resin was washed with DMF (3 x 2 mL, 1 min). The amino acid coupling was carried out by treatment with a solution of α -amino acids (4 equiv), *N*-HBTU (3.95 equiv) in DMF (2 mL), which was mixed with DIEA (0.195 M solution in DMF, 1.2 equiv) 1 min before the addition and the resulting mixture was shaken by bubbling Ar for 15 min. Finally, the resin was washed with DMF (3 x 2 mL, 1 min). The efficiency of each amino acid coupling and deprotection was monitored employing the TNBS test.^{S3}

For automated synthesis, a variant of the previous protocol was used instead, according to manufacturer's recommendations. 0.05 mmol of Rink Amide resin was placed into the peptide synthesiser reaction vessel, swollen in DMF, followed by cycles of Fmoc cleavage with piperidine 20% in DMF, washings (3 x 5 mL), then amino acid (5 equiv 2 M amino acid solution in DMF), DIC (10 equiv) and Oxyma (10 equiv) were added into the reaction vessel and microwaved for 5 min under temperature control followed by washings (3 x 5 mL). All steps were performed under nitrogen atmosphere. After the linear peptide was finished the resin was transferred to a different reaction vessel to perform the peptide modification manually.

Fluorophore coupling: the Fmoc-protecting group of the initial amino acid was removed by using a solution of piperidine in DMF (20%, 4 mL) for 15 min and the resin was washed with DMF (3 x 3 mL). The coupling was carried out by the addition of a solution of 5-carboxytetramethylrhodamine or 5,6-carboxyfluorescein (1 equiv), HATU (1 equiv), and DIEA (0.195 M, 1 equiv) in DMF (2 mL) and the mixture was stirred by bubbling Ar for 4 hours. Finally, the resin was washed with DMF (3 x 3 mL) and DCM (3 x 3 mL).

Acetylation in the N-terminus: Once the linear peptide was finished the acetylation capping of *N*-terminal group was performed by standard Fmoc deprotection conditions (20% piperidine in DMF (2 x 5 mL, 15 min) followed by treatment with a solution of 2,6-lutidine/acetic anhydride (1:1, 3 mL). The resin suspension was mechanically shaken for 40 min and washed with DMF (3 x 3 mL, 1 min) and DCM (3 x 3 mL, 5 min).

General protocol for peptide cleavage and purification: Finally, peptides were deprotected and cleaved from the resin by standard TFA cleavage procedure at rt by using the TFA/DCM/H₂O/TIS (90:5:2.5:2.5, 3 mL per 70 mg of resin) for 2 h. Then, the mixture was filtered, washed with TFA (1 mL) and the peptide was precipitated with ice-cold Et₂O (50 mL). The precipitate was centrifuged and dissolved in H₂O (5 mL) and purified by RP-HPLC. Finally, the corresponding fractions were lyophilised to afford the pure solid peptides.

2.3.1. Synthesis of peptide MP1

Following the general protocol of the SPPS, **MP1** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 30%. *R_t* 14.7 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→21 min)]. **MS** (ESI, H₂O): 1578.0 (6, [M+H]⁺), 789.6 (100, [M+2H]²⁺), 526.9 (71, [M+3H]³⁺). **HRMS** (ESI): Calcd for C₆₈H₁₂₅N₂₆O₁₇ [M+H]⁺: 1577.9702; found: 1577.9711. See Figure S15.

2.3.2. Synthesis of peptide CF-MP1

Following the general protocol of the SPPS followed by fluorophore coupling, **CF-MP1** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 9%. *R_t* 14.9 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)]. **MS** (ESI, H₂O): 947.8 (37, [M+2H]²⁺), 632.2 (100, [M+3H]³⁺), 474.4 (27, [M+4H]⁴⁺). **HRMS** (ESI): Calcd for C₈₇H₁₃₄N₂₂O₂₂ [M+2H]²⁺: 947.5100; found: 947.5078. See Figure S16.

2.3.3. Synthesis of peptide TM-MP1

Following the general protocol of the SPPS followed by fluorophore coupling, **TM-MP1** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 12%. *R_t* 15.2 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)]. Synthesis and characterization had been previously described as **P₈** in Pazo *et al.*^{S4}

2.3.4. Synthesis of peptide MP2

Following the general protocol of the SPPS, **MP2** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 10%. *R_t* 14.4 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)]. **MS** (ESI, H₂O): 1578.9 (5, [M+H]⁺), 789.6 (100, [M+2H]²⁺), 526.9 (77, [M+3H]³⁺). **HRMS** (ESI): Calcd for C₆₈H₁₂₄N₂₆O₁₇ [M+2H]²⁺: 1577.9716; found: 1577.9711. See Figure S17.

2.3.5. Synthesis of peptide P3

Following the general protocol of the SPPS, **P3** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 16%. *R_t* 15.4 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)]. **MS** (ESI, H₂O): 1081.1 (33, [M+2H]²⁺), 721.1 (100, [M+3H]³⁺), 541.1 (39, [M+4H]⁴⁺). **HRMS** (ESI): Calcd for C₉₂H₁₆₈N₃₆O₂₄ [M+2H]²⁺: 1080.6515; found: 1080.6511. See Figure S18.

2.3.6. Synthesis of peptide Arg8

Following the general protocol of the SPPS, **Arg8** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 58%. *R_t* 9.5 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)]. **MS** (ESI, H₂O): 882.9 (11, [M+2H+4TFA]²⁺), 826.2 (22, [M+2H+3TFA]²⁺), 769.0 (26, [M+2H+2TFA]²⁺), 712.3 (17, [M+2H+TFA]²⁺), 589.2 (30.3, [M+3H+4TFA]³⁺), 551.2 (78, [M+3H+3TFA]³⁺), 513.1 (100,

[M+3H+2TFA]³⁺, 475.1 (77, [M+3H+TFA]³⁺), 437.1 (48, [M+3H+TFA]³⁺). **HRMS** (ESI): Calcd for C₅₀H₁₀₃N₃₃O₉ [M+2H]²⁺: 654.9304; found: 654.9303. See Figure S19.

2.3.7. Synthesis of peptide GALA

Following the general protocol of the SPPS, **GALA** was obtained after RP-HPLC purification [Phenomenex Luna C18(2) 100A column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→5 min), 95:5→5:95 (5→35 min)] with an overall yield of 0.5%. *R_t* 20.6 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)]. **MS** (ESI, H₂O): 1528.8 (46, [M+2H-OH]²⁺), 1537.2 (17, [M+2H]²⁺), 1025.3 (100, [M+3H]³⁺), 769.2 (16, [M+4H]⁴⁺). **HRMS** (ESI): Calcd for C₁₃₈H₂₂₀N₃₄O₄₅ [M+2H]²⁺: 1536.7981; found: 1536.7980. See Figure S20.

2.3.8. Synthesis of peptide L17E

L17E was synthesized and purified as previously described,^{S5} with an overall yield of 23.3%. *R_t* 10.6 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)]. **MS** (ESI, H₂O): 1430.2 (23, [M+2H]²⁺), 953.9 (100, [M+3H]³⁺), 715.8 (26, [M+4H]⁴⁺), 572.95 (7, [M+5H]⁵⁺). See Figure S21.

2.3.9. Synthesis of Pep-1

Pep-1 was synthesized and purified as previously described,^{S6} with an overall yield of 3.5%. *R_t* 11.6 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)]. **MS** (ESI, H₂O): 1424.2 (15, [M+2H]²⁺), 949.9 (100, [M+3H]³⁺), 712.7 (20, [M+4H]⁴⁺), 570.8 (13, [M+5H]⁵⁺). See Figure S22.

2.3.10. Synthesis of dfTAT

dfTAT was synthesized and purified as previously described,^{S7} with an overall yield of 8%. *R_t* 11.5 min [RP-HPLC Agilent SB-C18 column, H₂O (0.1% TFA)/ CH₃CN (0.1% TFA) 95:5→5:95 (0→20 min)]. **MS** (ESI, H₂O): 1588.0 (10, [M+3H+6TFA]³⁺), 1550.2 (10, [M+3H+5TFA]³⁺), 1162.9 (75, [M+4H+5TFA]⁴⁺), 1134.4 (100, [M+4H+4TFA]⁴⁺), 1105.8 (61, [M+4H+3TFA]⁴⁺), 884.9 (72, [M+5H+3TFA]⁵⁺), 862.1 (55, [M+5H+2TFA]⁵⁺), 699.5 (50, [M+6H+TFA]⁶⁺), 680.75 (45, [M+6H]⁶⁺), 599.75 (16, [M+7H+TFA]⁷⁺). See Figure S23.

2.4. Liposome preparation

The procedure for liposome preparation has been previously reported.^{S8} Briefly, a thin lipid film was prepared using 1 mL of 25 mg/mL of lipid in chloroform and evaporating the chloroform on a rotary evaporator and then *in vacuo* overnight. The lipids were resuspended with one of the intravesicular solutions described below for 45 min. The resulting vesicles were extruded 11 times through a 0.1 μm Nucleopore polycarbonate filter. Extravesicular components were removed by desalting column (Sephadex G-50) pre-equilibrated with extravesicular buffer. The final concentration of vesicles is 5 mM.

In the case of ANTS/DPX – encapsulated LUVs (LUVs_{enc}ANTS/DPX) the extravesicular buffer is composed by 10 mM MES and 150 mM NaCl, at pH 7.4 or 5.5, and the intravesicular buffer is composed by 10 mM MES, 70 mM NaCl, 12.5mM ANTS and 45 mM DPX at pH 7.5 or 5.5 respectively. The lipid composition is 100% POPC or POPC/POPG at 3:1 molar ratio.

In order to make NBD-labelled lipid vesicles, lipids labelled in the polar head (PE-NBD) or in the hydrophobic tail (NBD-PE) are added to the lipid mixture as 0.5%. The remaining lipid composition is 99.5% POPC or POPC/POPG molar ratio 3:1. The intra and extravesicular buffer are the same (10 mM MES and 150 mM NaCl, at pH 7.5 or 5.5). In this case, the desalting column (Sephadex G-50) step is not necessary.^{S9}

In the case of non-fluorescent LUVs, the intra and extravesicular buffers are composed by 10 mM MES and 150 mM NaCl, at pH 7.5 or 5.5 respectively, and the lipid composition is 100% POPC or POPC/POPG molar ratio 3:1. In this case, the desalting column (Sephadex G-50) step is omitted.

Dextran loaded liposomes (LUVs \subset Dextran) were prepared following the protocol described by Sani *et al.*^{S10} First, the film lipid was formed with POPC or POPC/POPG 3:1 by rotary evaporation and then *in vacuo* overnight. Thus, the lipid was rehydrated with a solution of 20 mM of 10 kDa FITC-dextran and 20 mM of 40 kDa TM-Dextran, 10 mM MES, 5 mM NaCl at two different pH (7.5 and 5.5). After that, the resulting mixture was extruded 11 times through a 0.2 μ m Nucleopore polycarbonate filter. In order to remove the extravesicular components, the samples were centrifuged three times for 40 min, 1500 rpm at 10 °C. After each centrifugation the supernatant was removed and replaced by fresh extravesicular buffer (10 mM MES, 7 mM NaCl) at the corresponding pH.

2.5. ANTS/DPX assay

LUVs \subset ANTS/DPX stock solutions (50 μ L) were diluted with a buffer (10 mM MES and 150 mM NaCl, at pH 7.4 and 5.5), placed in a thermostated fluorescence cuvette (25 °C) and gently stirred (total volume \sim 2000 μ L; final lipid concentration \sim 125 μ M). ANTS efflux was monitored at λ_{em} 510 nm (λ_{ex} 353 nm) as a function of time after addition of **MP1** at $t = 50$ s and aqueous Triton X-100 (1.2%, 20 μ L, 185 μ M final concentration) at $t = 225$ s; each measurement was made in triplicate. Fluorescence intensities were normalized to fractional emission intensity $I(t)$ using equation (S1).

$$I(t) = (I_t - I_0) / (I_\infty - I_0) \quad (S1)$$

where I_0 is the initial intensity of the vesicles alone, $I_\infty = I_t$ at saturation after lysis. Effective concentration for peptides (EC_{50}) and Hill coefficient (n) were determined by plotting the fractional activity $Y (= I(t)$ at saturation just before lysis, $t = \sim$ 200 s) as a function of **MP1** $c_{Peptide}$ and fitting them to the Hill equation (S2).

$$Y = Y_0 + (Y_{MAX} - Y_0) / \{1 + (EC_{50} / c_{Peptide})^n\} \quad (S2)$$

where Y_0 is Y before the addition of the peptide, Y_{MAX} is Y with an excess of peptide at saturation.

For reversible pore gating experiments, LUVs \subset ANTS/DPX (3:1 molar ratio POPC:POPG) stock solutions (50 μ L) were diluted with a buffer (10 mM MES and 150 mM NaCl, at pH 7.4 and 5.5) and placed in a thermostated fluorescence cuvette (25 °C) and gently stirred (total volume \sim 2000 μ L; final lipid concentration \sim 125 μ M). ANTS efflux was monitored at λ_{em} 510 nm (λ_{ex} 353 nm) as a function of time after addition of **MP1** (10 μ M) at $t = 25$ s peptide addition, $t = 50$ s lipid addition (POPG, 50 μ M), $t = 150$ s peptide addition, $t = 175$ s lipid addition, $t = 275$ s peptide addition, $t = 300$ s lipid addition. Finally, an aqueous Triton X-100 solution (1.2%, 20 μ L, 185 μ M final concentration) was added at $t = 475$ s.

2.6. Dextran release in vesicles

LUVs \subset Dextran stock solutions (25 μ L) were diluted with a buffer (10 mM MES and 7 mM NaCl, at either pH 7.4 or 5.5) and placed in an eppendorf. Then the minimal peptide **MP1** was added at different concentrations and stirred for 30 min (total volume \sim 1000 μ L; final lipid concentration \sim 125 μ M). The mixture was centrifuged for 35 min at 10 °C and 1500 rpm. The supernatant was collected and measured by spectrofluorometry: FITC-dextran (10 kDa) was monitored at λ_{em} 500 - 650 nm (λ_{ex} 492 nm), TM-dextran (40 kDa) was monitored at λ_{em} 560 - 700 nm (λ_{ex} 555 nm). Fluorescence intensities were normalized to fractional emission intensity $I(t)$ using equation (S1)

$$I(t) = (I_t - I_0) / (I_\infty - I_0) \quad (S1)$$

where I_0 is I_t of vesicles alone, $I_\infty = I_t$ at saturation after lysis. Effective concentration for peptides (EC_{50}) and Hill coefficient (n) were determined by plotting the fractional activity $Y (= I(t)$ at saturation just before lysis) as a function of peptide concentration c_{Peptide} and fitting them to the Hill equation (S2).

$$Y = Y_0 + (Y_{\text{MAX}} - Y_0) / \{1 + (EC_{50} / c_{\text{Peptide}})^n\} \quad (\text{S2})$$

where Y_0 is Y without peptide, Y_{MAX} is Y with an excess of **MP1** at saturation.

2.7. Circular Dichroism

Circular dichroism measurements were carried out with the following settings: acquisition range: 300-190 nm; bandwidth: 1.0 nm; accumulation: 3 scans; data pitch: 1 nm; CD scale 200 mdeg/1.0 dOD; D.I.T. (Data Integration Time): 1 s; scanning mode: continuous; scanning speed: 200 nm/min. Measurements were done at 10 °C and 40 °C in a quartz cell of 0.2 cm path length at a final volume of 0.5 mL (buffer 10 mM MES, 150 mM NaCl at two pH: 7.4 and 5.5) with a final peptide concentration of 100 μM. The results are expressed as the mean residue molar ellipticity $[\theta]_{\text{MRt}}$ with units of degrees·cm²·dmol⁻¹ and calculated using the equation S3. The percentage of helicity was calculated using the equation S4.^{S11} As this value is used for comparisons among similar peptides in different environments, no correction for the number of aromatic aminoacids is required. For the measurements in presence of non-fluorescent LUVs (100% POPC or POPC/POPG at molar ratio 3:1), samples were prepared by mixing the peptides at 100 μM and different amounts of liposomes at different lipid:peptide ratios (1.25, 5, 12, 50). For each sample, a blank measurement was done prior addition of the peptide, and subtracted to the other measurements before analysis.

$$[\Theta]_{\text{MRt}} = \frac{0.1 \times \theta}{C \cdot l \cdot \text{number of residues}} \quad (\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}) \quad (\text{S3})$$

Equation S3: Formula to calculate the ellipticity. Θ is the ellipticity (mdeg), C is the peptide concentration (M) and l is the cell path length (cm).

$$[\Theta]\% = \frac{|\Theta_{\text{MRt}_{222}} - 2340|}{30300} \times 100 \quad (\text{S4})$$

Equation S4: Formula to calculate the percent of helicity in which the molar ellipticity at 222 nm is an absolute value.^{S11}

2.8. DLS experiments

Non-fluorescent LUVs (50 μL, 125 μM) (POPC or POPC/POPG 3:1) were added to a buffer solution (10 mM MES and 150 mM NaCl, at pH 7.5 and 5.5) to a final volume of 200 μL (20 °C, duration = 70 s). The best parameters were found using the automatic mode, after which the attenuator index was fixed at 6. Measurements were done after the addition of increasing amounts of the peptide (50, 100, 200, 400, 800 μM) to the sample, and ended after vesicle lysis with Triton X-100, when the equipment could not measure, as the counted photons are 0. The standard deviation is given by equation S5.^{S12}

$$\text{PdI} = \left(\frac{\text{SD}}{\text{Mean}} \right)^2 \quad (\text{S5})$$

Equation S5: Formula to calculate the standard deviation using the mean and polydispersity index (PdI).

2.9. FRET and fluorescence experiments

The minimal peptide **MP1** was modified at the N-terminus with either 5-TAMRA or 5-carboxyfluorescein, which constitute a FRET pair, to achieve the peptides **TM-MP1** and **CF-MP1** respectively. The emission of the donor carboxyfluorescein (at $\lambda_{\text{ex}} = 494$ nm, $\lambda_{\text{em}} = 518$ nm) was measured in a final volume of 2 mL containing a mixture of 50 μL, 125 μM vesicles and 2.5 μM **CF-MP1**. The peptide FRET partner **TM-MP1** 2.5 μM (5μM final concentration of both **CF-MP1** and **TM-**

MP1) was added at $t = 25$ s and the fluorescence monitored until $t = 200$ s. **CF-MP1** emission was determined at $t = 10$ s and $t = 150$ s and FRET efficiency calculated according equation S6.

$$E = 1 - (F_{DA}/F_D) \quad (S6)$$

Equation S6: Formula to calculate the Efficiency (E), F_{DA} is the fluorescence of the donor in the presence of the acceptor; F_D fluorescence of the donor alone.

The second FRET experiment was performed using the same peptide labelled at the N-terminus with TAMRA (**TM-MP1**). Anionic and neutral vesicles containing 0.5% NBD-PE or PE-NBD were diluted (50 μ L in 2 mL) in MES buffer at two different pH (7.5 and 5.5). The emission of the donor NBD ($\lambda_{ex} = 470$ nm, $\lambda_{em} = 531$ nm) was measured at $t = 0$ s and after the addition of peptide acceptor pair (0.05, 0.1, 0.5, 1 μ M) from $t = 25$ s until $t = 200$ s. NBD emission values at $t = 10$ s and $t = 150$ s were used for FRET efficiency calculation using equation S6.

Emission fluorescence spectra was also measured at different concentrations of **CF-MP1** ($\lambda_{ex} = 494$ nm) in aqueous solution (MES buffer, pH 7.5). Quenching effect at increasing concentrations suggests a micelle-like aggregation at concentrations over 1.5 μ M.^{S13} The shift of the peak of maximum fluorescence intensity indicates a complete aqueous solvating effect in peptide molecules at concentration lower than 1.5 μ M.^{S14}

2.10. Computational methods

Since it is generally believed that early endosomes share the same overall lipid composition as the plasma membrane, as they are both part of the same recycling territory,^{S15} this study used an asymmetric human erythrocyte membrane model, following the study of Tian *et al.*^{S16}, which involves three lipid types, including dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylethanolamine (DPPE), and dipalmitoyl phosphatidylserine (DPPS). In the outer leaflet of the membrane, the lipid ratio is 9:1 for DPPC and DPPE; in the inner membrane leaflet, the lipid ratio is 3:5:2 for DPPC, DPPE, and DPPS. Mapping of lipid molecules was based on the Martini CG force field (Martini_v2.2).^{S11,S12} Choline groups of lipids are represented by the CG beads Qo, Qd, and P5 for DPPC, DPPE, and DPPS, respectively. The phosphate groups of all lipids are represented by Qa. The glycerol groups and carbon tails of all lipids are represented by Na and C1, respectively.

The peptide **MP1** model was built following the extended Martini force field for proteins using the martine.py script.^{S11,S13} Peptide **MP1** contains 3 Leu residues, 3 Arg residues and 10 Ala residues, so that the total charge of each unit is +3. The terminal edges of the peptides were not charged.

A random distribution of 31 units of peptide **MP1** interaction of an asymmetric membrane composed by 1309 CG lipids was investigated, with an initial size of the box of 20 x 20 x 15 nm. After a short equilibration, long MD simulations of 1000 ns for each case were performed in absence and presence of an external electric field of 0.03 V/nm applied across the bilayer. The system temperature was kept at 310 K using a V-rescale thermostat,^{S20} and periodic boundary conditions were used in all simulations. The Parrinello-Rahman barostat^{S21} with semisotropic pressure was applied. The van der Waals interaction cutoff was 1.1 nm. In the case of atomistic resolution, AT-MD simulations were carried out using the Gromos54a7 force field.^{S22} The edges of peptide **MP1** were modeled as ACE and NH2, respectively. The box was solvated with water or TFE, respectively. The system temperature was kept at 310 K using a V-rescale thermostat,^{S20} and periodic boundary conditions were used in all simulations. The Parrinello-Rahman barostat^{S21} with isotropic pressure was applied. The van der Waals interaction cutoff was 1.0 nm. Particle mesh Ewald summation (PME) method^{S23} was used to determine electrostatic interactions. All simulations were performed by the GROMACS 4.5.4 package^{S24} and results were represented by Visual Molecular Dynamics (VMD) 1.9 software.^{S25}

2.11. Planar lipid bilayer recordings

Experiments were performed in planar lipid bilayer membranes (BLMs) formed by the Montal and Rudin technique.^{S26} The recordings were carried out by using bilayers of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) across a 80-100 μ m diameter hole of a 25 μ m thick PTFE (Goodfellow Corporation, Malvern, PA, USA). Bilayers were formed by first pretreating the aperture with a drop of 1% (v/v) hexadecane in n-pentane. The buffer solution (0.5 M of KCl and 10 mM of MOPS) was added to both 1 mL chambers, then, DPhPC in n-pentane (5 μ L, 5 mg/mL) was added to both wells; after that, the solvent evaporated. A bilayer was formed when the electrolyte was lowered and raised, bringing the

two lipid surface monolayers together at the aperture. The capacitance of the bilayer was checked and the stability of the bilayer was monitored for 5 min at +200 mV, observing no fluctuations in the current. After planar membrane formation the peptide dissolved in MQ water was added to both chambers at 25 μ M. The whole BLM conductance was measured at voltage-clamp-mode-holding potential from trans to cis chamber. Raw ion current recording was acquired at 1 kHz at a sampling frequency of 5 kHz. The current was amplified by using an Axopatch 200B amplifier (Molecular Devices), digitized with a Digidata 1440 A (Molecular Devices) converter, while data collection was done by the Clampex 10.7 software (Molecular Devices). Data processing and analysis was carried out with Clampfit 10.7 (the resulting current was filtered using Low pass Bessel (8-pole)). All Axons instruments and software are products of Molecular Devices (Sunnyvale, CA). According to the pore conductance, the peptide pore size was calculated following the Hille equation (S7).^{S27} The conductivity of 0.5 M KCl 10 mM MOPS at 25 °C is 5.1 S·m⁻¹, r the radius and l the length of the pore (4.7 nm) (theoretical thickness of DPhPC membrane). From the single channel electrical recordings, the current of the open state is around 1,100 pA and the estimated diameter is around 3.16 nm; for long opened states is around 1,900 pA and the diameter around 4.4 nm.

$$\frac{1}{g} = \left(l + \frac{\pi r}{2} \right) \cdot \left(\frac{\rho}{\pi r^2} \right) \quad (\text{S7})$$

Equation S7. Hille equation. g is the pore conductance; l the length of the pore, ρ the resistivity of the recording solution and r the inner radius of the pore.

2.12. Cell culture experiments

2.12.1. Cell lines and culture

HeLa, Vero, and A549 cells were grown in the same conditions: at 37 °C, 5% CO₂, in Dulbecco's Modified Eagle's Medium (DMEM; 4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate), supplemented with 10% fetal bovine serum and 1% of Penicillin-Streptomycin-Glutamine Mix.

Human retinal pigmentary epithelium ARPE-19 cell line was maintained on Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate) supplemented with 10% FBS and 1% Penicillin-Streptomycin-Glutamine Mix (Fisher) at 37 °C, 5% CO₂ in an INCO 108 incubator (Memmert).

To generate a Cre Reporter-expressing HeLa cell line, HeLa cells were transfected with the plasmid Cre Reporter (a gift from Niels Geijsen; Addgene plasmid # 62732).^{S28} In this plasmid, the gene for dsRED is flanked by two *loxP* sites and followed by EGFP gene. In the absence of recombination, the stop codon from dsRED prevents EGFP translation, while Cre-mediated recombination removes the dsRED gene, switching the expression from the red fluorescent protein to the green fluorescent protein. Stable transfectants were selected by maintaining the cell culture in presence of 0.5 μ g/mL puromycin (Enzo Life Sciences).

2.12.2. Cellular uptake and endosomal release of dextran

For live cell imaging, cells grown on glass bottom dishes were washed with DMEM stock and incubated for 1 h with a solution of peptide and dextran, using different concentrations of **MP1** and 0.25 mg/mL 10 kDa Alexa₄₈₈-Dextran, 0.25 mg/mL 10 kDa TM-Dextran, 1 mg/mL of 40 kDa FITC-Dextran, or 0.25 mg/mL of 70 kDa FITC-Dextran. In co-incubation experiments, 0.25 mg/mL 10 kDa TM-Dextran and 1.25 mg/mL 70 kDa FITC-Dextran were used. After that, cells were washed with DMEM twice and incubated for 3 h with DMEM supplemented with 10% FBS. Finally, cells were washed with DMEM without phenol red and observed with a spinning disk confocal microscope. For lysosomal labelling, cells were incubated for 30 min with 100 nM of LysoTracker Deep Red after the 3 h incubation, and washed before imaging.

For the experiments in the presence of endocytosis inhibitors, HeLa cells were seeded the day before in 8-well micro-slides (ibiTreat, Ibidi) at 30000 cells per well, washed and treated with chlorpromazine (30 μ M), dynasore (80 μ M), methyl- β -cyclodextrin (5 mM) or EIPA (50 μ M) diluted in DMEM without

serum. Hoechst 33342 (1 μM) was added to stain nuclei. After 30 min of incubation, this solution was replaced by 250 μL of DMEM without serum containing 30 μM of **MPI**, 0.25 mg/mL 10 kDa TM-Dextran and the same amount of the corresponding inhibitor. After incubation for 1 h at 37 $^{\circ}\text{C}$, cells were washed twice with DMEM without phenol red and imaged at the confocal microscope. The number of cells with cytosolic dextran was counted for more than 300 cells for each condition, and experiments were done three times. Data were normalized to untreated control (100%).

For the comparison of dextran endosomal release in the presence and in the absence of serum, HeLa cells were incubated for 1 h with a solution of 0.25 mg/mL 10 kDa Alexa₄₈₈-Dextran and various concentrations of **MPI** in DMEM stock or in DMEM supplemented with 10% FBS. After that, cells nuclei were stained with 1 μM Hoechst 33342 in DMEM without phenol red for 30 min, then cells were washed with DMEM without phenol red and incubated for 2.5 hours with DMEM without phenol red provided with 10% FBS. Finally, cells were observed with a Dragonfly confocal spinning-disk microscope.

2.12.3. Cell viability assay (MTT)

HeLa, Vero, A549 and ARPE-19 cells were incubated with different concentrations of **MPI** (0, 1, 2, 4, 8, 16, 32, 62.5, 125, 250, 500, 1000 μM) in DMEM for 1 h, then the solutions were removed and replaced with complete medium. After 24 hours, cells were incubated for 3 hours with 0.5 mg/mL MTT (tetrazolium salt), then the medium was removed and the formazan salt formed solubilized with DMSO. Another set of cells was directly incubated with medium containing MTT for 1 h immediately after the initial 1 h incubation, to study the viability in the absence of recovery. Cells viability was then quantified by absorbance at 570 nm with an Infinite 200 PRO microplate reader (Tecan). This viability test measures the reduction of MTT to formazan by mitochondrial activity. Data was normalized to untreated cells (100% viability), after blank subtraction (Triton X-100 treated cells).

2.12.4. Saporin experiments

For the saporin experiments, HeLa cells were incubated in a 96-well plate with 0 or 10 $\mu\text{g/mL}$ of saporin and increasing concentrations of the **MPI**, **Args**, and **GALA** acetylated peptides. Cells were co-incubated with protein and peptide for 1 h in culture medium without serum or antibiotics, and then the compounds were removed, cells were washed and complete medium was added. Cells were further incubated for 6 h or 24 h before the addition of MTT tetrazolium salt at a final concentration of 0.5 mg/mL. For the more resistant A549 cells, this incubation was 24 h before adding MTT. Cells were further incubated for 3 h, and the medium removed before solubilizing the formazan salt with DMSO. Cells viability was then quantified by absorbance. Data was normalized to untreated cells (100% viability), after blank subtraction (Triton X-100 treated cells).

2.12.5. Cre recombinase expression and purification

Escherichia coli BL21(DE3)-Codon Plus cells (Stratagene) were transformed with the plasmid pET-Cre recombinase (a gift from Niels Geijsen; Addgene plasmid #62730).^{S28} A bacterial preculture grown overnight at 37 $^{\circ}\text{C}$ was diluted 1:250 in LB medium supplemented with 100 $\mu\text{g/mL}$ Ampicillin. Cultures were incubated at 37 $^{\circ}\text{C}$ until OD₆₀₀ ~ 0.8 and induced with 1 mM IPTG for 16 h at 16 $^{\circ}\text{C}$. Cells were harvested by centrifugation, pellets were resuspended in 1:50 volume of lysis buffer (600 mM NaCl, 20 mM Tris pH 7.5, 20 mM imidazole, 1 mg/mL lysozyme supplemented with protease inhibitors without EDTA) and sonicated on ice 15 x 10 s (Cycle 0.5, Amplitude 70%) with a UP200S ultrasonic processor (Hielscher). Cellular debris was removed by centrifugation at 16000 g 15 min and the supernatant was incubated with HisPur Ni-NTA resin (ThermoFisher) for 1 h at 4 $^{\circ}\text{C}$, washed 3 times with 3 volumes of binding buffer (500 mM NaCl, 20 mM Tris pH 7.5, 5 mM imidazole) and then 2 times with wash buffer (500 mM NaCl, 20 mM Tris pH 7.5, 60 mM imidazole). The protein was eluted with several incubations of elution buffer (500 mM NaCl, 20 mM Tris pH 7.5, 500 mM imidazole). Imidazole from the fractions containing the protein of interest was removed by several washes on an Amicon UFC (Millipore) and finally, protein was concentrated in storage buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 20% glycerol). Aliquots were snap-frozen and stored at -80 $^{\circ}\text{C}$.

2.12.6. Cre recombinase delivery experiments

Cells were seeded on round coverslips (12 mm diameter) in 24-well plate at a density of 75000 cells/well the day prior the experiment. Cre recombinase and peptide complexes were prepared as 5X

solutions in DMEM and added to the cells to the final working concentration (1 μM Cre, and 2.5, 5, 10 and 20 μM peptide). Cells were incubated with the complexes for 4 h, and then the complexes were removed and substituted with complete culture medium. After 72 h, cells were processed for epifluorescence microscopy. After incubation with 1 μM Hoechst 33 342 in complete medium for 30 min, cells were washed twice with PBS, incubated 30 min with 4% paraformaldehyde (PFA) in PBS and washed again with PBS, then the coverslips were removed from the 24-well plate, placed onto a glass slide and mounted with Mowiol 4-88 mounting medium (Calbiochem).

For quantification by flow cytometry, cells were seeded in a 24-well plate, and incubated as stated for the microscopy experiment. After 72 h, cells were trypsinized, trypsin was neutralized with PBS supplemented with 2% FBS and 0.5 mM EDTA, and the cell suspension was transferred to a 96-well plate. Cells were analysed by flow cytometry with a Guava EasyCyte BG HT, and the results were processed with InCyte software (GuavaSoft 3.2, Millipore). Cre recombinase delivery was assessed by calculating the percentage of EGFP expressing cells over the total of dsRED- and EGFP-expressing cells, after gating for cells with typical FSC and SSC parameters.

2.12.7. Antibody delivery in cell culture

HeLa or ARPE-19 cells were seeded in glass-bottom black 96-well plates (CellVis). Cells were incubated with mouse monoclonal anti-nuclear pore complex proteins antibody (Mab414), using 0.3 mg/mL for HeLa cells and 0.15 mg/mL for ARPE-19 cells, and several concentrations of peptide **MP1**, diluted in cell culture medium without FBS. After 1 h of incubation, cells were extensively washed and cell culture medium supplemented with 10% FBS was added for a further 2 h incubation. Cells were again washed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min, washed twice with PBS permeabilized with 0.5% Triton X-100 in PBS for 3 min. Cells were incubated in blocking solution (2% BSA in PBS) for 30 min, and then incubated for another 30 min with 1:1000 dilution of goat anti-mouse IgG conjugated with Alexa Fluor 594 (Invitrogen, #A11012) and 0.5 $\mu\text{g}/\text{mL}$ DAPI. Finally, cells were washed with PBS and observed with a confocal microscope.

2.12.8. CF-labelling and delivery of IgG

IgG-CF was prepared by the reaction of human IgG (4 mg in 200 μL of PBS pH 7.2) with Carboxyfluorescein-NHS (50 equiv) in NaHCO_3 buffer (pH 9) at room temperature for 2 h. The resulting solution was dialysed overnight in PBS buffer with 20% NaHCO_3 at 4°C. To quantify the number of carboxyfluorescein per IgG antibody the absorbance of the mixture was measured using a NanoDrop UV-Vis Spectrophotometer. Absorbance values at 280 nm (maximum absorbance for antibody) and 492 nm (maximum absorbance for the carboxyfluorescein) were measured for various dilutions of the dialysed IgG-CF. Concentrations of antibody and carboxyfluorescein were calculated and the relation between both concentrations gave a value of around 8 CF per antibody. IgG-CF was diluted at 1 mg/mL in DMEM without FBS in the absence or presence of 30 μM **MP1**, and this mixture was added to HeLa cells. After 1 h of incubation, cells were washed and further incubated for 90 min with DMEM supplemented with 10% FBS, before confocal imaging.

2.12.9. Purification and delivery of GST-NLS-GFP

Escherichia coli BL21(DE3)-Codon Plus cells (Stratagene) were transformed with the plasmid pGEX-SV40NLS-GFP^{S29} and grown in LB containing ampicillin (100 $\mu\text{g}/\text{mL}$) until OD600 \sim 1. Protein expression was induced with 0.5 mM IPTG at 20 °C for 16 h. Cells were harvested and resuspended in PBS containing 1% Triton X-100 and protease inhibitors and sonicated on ice with a UP200S ultrasonic processor (Hielscher). After centrifugation (12000 g, 15 min, 4 °C), the supernatant was filtered and incubated for 40 min with Glutathione Sepharose 4B resin (GE Healthcare). After 3 washes with PBS, protein was eluted from the resin with 10 mM glutathione in 50 mM Tris-HCl (pH 8), concentrated to 80 μM and stored at -80 °C. The protein obtained is a fusion protein with the glutathione-S-transferase tag followed by SV40 large T-antigen NLS sequence (PKKKRKVEDP) and EGFP, with a theoretical molecular weight of 55 kDa and an isoelectric point of \sim 6. Delivery assays were performed by incubation of HeLa cells with 10 μM GST-NLS-GFP alone or in combination with 30 μM **MP1** for 1 h in DMEM without FBS. Cells were washed and incubated for 90 min in DMEM supplemented with 10% FBS and observed with a confocal microscope.

2.13. *Ex vivo* cornea experiments

2.13.1. Murine corneas culture

Mice (Sfl-Cre, The Jackson Laboratory) were treated and sacrificed according to the guidelines for the good use of laboratory animals in effect at the animal facility of the Center for Research in Molecular Medicine and Chronic Diseases (CiMUS). Mice were sacrificed by an intraperitoneal injection of a fatal dose of ketamine/xylazine. Eviscerated murine eyes were maintained in warm Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich) while the corneas were removed from the eye bulbs and separated from the iris using surgical forceps and scissors. The corneas were maintained in warm HBSS and immediately used for the experiments. From each mouse, one cornea was used for the immunofluorescence and one to assess the toxicity of the complexes.

2.13.2. Antibody delivery in cornea

The intracellular delivery of anti-nuclear pore complex proteins antibody Mab414 in murine corneas was assessed preparing solutions 2X of antibody (where the final concentration 1X was 0.3 mg/mL) and **MP1** peptide in Ames' medium. 25 μ L of antibody solution were placed in a 96-well glass bottom black plate (CellVis) and corneas were placed in this solution, then the peptide solutions were added to the wells and the plate was incubated for 1 h at 37 °C, 5% CO₂, 95% humidity. After that, the corneas were washed with Ames' medium and incubated with Ames' medium supplemented with 10% FBS at 37 °C, 5% CO₂, 95% humidity. After 2 h the corneas were washed twice with PBS, incubated 30 min with 4% paraformaldehyde (PFA) in PBS, washed twice again and permeabilized with 0.5% Triton X-100 in PBS for 3 min. To prevent non-specific binding of the secondary antibody, blocking was performed by incubating the corneas in 2% BSA in PBS for 40 min, and then they were incubated for 40 min with a solution of 2% BSA, goat anti-mouse IgG conjugated with Alexa Fluor 594 (Invitrogen, #A11012), diluted 1:1000, and 0.5 μ g/mL DAPI. Finally, the corneas were washed twice with PBS and covered with PBS for imaging with a Dragonfly confocal spinning-disk microscope.

2.13.3. Live/Dead Staining

To assess corneas viability after the delivery of Mab414 antibody, corneas were incubated with 3.75 μ g/mL fluorescein diacetate (Sigma-Aldrich), and 4 μ g/mL propidium iodide (Sigma-Aldrich) in Ames' medium for 4 min in the dark, while gently shaking, then washed twice with PBS and immediately imaged with a confocal spinning-disk microscope.

2.14. Sources for Figure 6 table

Dextran properties were extracted from the manufacturer's specification sheets (Sigma-Aldrich and Fisher Scientific).

Protein structures for saporin (1QI7),^{S30} Cre recombinase (1CRX),^{S31} and antibody (1HZH),^{S32} were retrieved from pdb (rcsb.org)^{S33} and processed with Chimera.^{S34} Dimensions were calculated using Chimera from the diameters of the inertia ellipsoid of each protein.

Hydrodynamic radius for Cre recombinase^{S35} and antibody^{S36} were obtained from the indicated references.

pI/MW were calculated from the aminoacid sequences using Compute pI/MW tool from ExPASy.^{S37}

3. Supporting References

- (S1) J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona, *Nat. Methods*, 2012, **9**, 676–682.
- (S2) R. Behrendt, P. White and J. Offer, *J. Pept. Sci.*, 2016, **22**, 4–27.
- (S3) C. Kay, O. E. Lorthioir, N. J. Parr, M. Congreve, S. C. McKeown, J. J. Scicinski and S. V Ley, *Biotechnol. Bioeng.*, 2000, **71**, 110–8.
- (S4) M. Pazo, M. Juanes, I. Lostalé-Seijo and J. Montenegro, *Chem. Commun.* 2018, **54**, 6919–6922.
- (S5) M. Akishiba, T. Takeuchi, Y. Kawaguchi, K. Sakamoto, H.-H. Yu, I. Nakase, T. Takatani-Nakase, F. Madani, A. Gräslund and S. Futaki, *Nat. Chem.*, 2017, **9**, 751–761.
- (S6) M. C. Morris, J. Depollier, J. Mery, F. Heitz and G. Divita, *Nat. Biotechnol.*, 2001, **19**, 1173–1176.
- (S7) A. Erazo-Oliveras, K. Najjar, L. Dayani, T.-Y. Wang, G. A. Johnson and J.-P. Pellois, *Nat. Methods*, 2014, **11**, 861–867.
- (S8) J. M. Priegue, J. Montenegro and J. R. Granja, *Small*, 2014, **10**, 3613–3618.
- (S9) A. Kashiwada, M. Tsuboi, N. Takamura, E. Brandenburg, K. Matsuda and B. Kocsch, *Chem. - A Eur. J.*, 2011, **17**, 6179–6186.
- (S10) M. A. Sani, T. C. Whitwell, J. D. Gehman, R. M. Robins-Browne, N. Pantarat, T. J. Attard, E. C. Reynolds, N. M. O'Brien-Simpson and F. Separovic, *Antimicrob. Agents Chemother.*, 2013, **57**, 3593–3600.
- (S11) Y.-H. Chen and J. T. Yang, *Biochem. Biophys. Res. Commun.*, 1971, **44**, 1285–1291
- (S12) Malvern Instruments Limited. Application of Dynamic Light Scattering (DLS) to Protein Therapeutic Formulations: Principles, Measurements and Analysis - 3. DLS Deconvolution Algorithms. Whitepaper, 2014.
- (S13) N. O. Mchedlov-Petrosyan, T. A. Cheipesh, A. D. Roshal, A. O. Doroshenko and N. A. Vodolazkaya, *Methods Appl. Fluoresc.*, 2016, **4**, 034002.
- (S14) F. M. Zehentbauer, C. Moretto, R. Stephen, T. Thevar, J. R. Gilchrist, D. Pokrajac, K. L. Richard and J. Kiefer, *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.*, 2014, **121**, 147–151.
- (S15) C. Bissig and J. Gruenberg, *Cold Spring Harb. Perspect. Biol.*, 2013, **5**, a016816.
- (S16) W. Tian and Y. Ma, *Soft Matter*, 2012, **8**, 6378.
- (S17) D. H. de Jong, G. Singh, W. F. D. Bennett, C. Arnarez, T. A. Wassenaar, L. V. Schäfer, X. Periole, D. P. Tieleman and S. J. Marrink, *J. Chem. Theory Comput.*, 2013, **9**, 687–697.
- (S18) S. O. Yesylevskyy, L. V. Schäfer, D. Sengupta and S. J. Marrink, *PLoS Comput. Biol.*, 2010, **6**, e1000810.
- (S19) L. Monticelli, S. K. Kandasamy, X. Periole, R. G. Larson, D. P. Tieleman and S.-J. Marrink, *J. Chem. Theory Comput.*, 2008, **4**, 819–834.
- (S20) G. Bussi, T. Zykova-Timan and M. Parrinello, *J. Chem. Phys.*, 2009, **130**, 074101.
- (S21) M. Parrinello and A. Rahman, *J. Appl. Phys.*, 1981, **52**, 7182–7190.
- (S22) N. Schmid, A. P. Eichenberger, A. Choutko, S. Riniker, M. Winger, A. E. Mark and W. F. van Gunsteren, *Eur. Biophys. J.*, 2011, **40**, 843–856.
- (S23) U. Essmann, L. Perera, M. L. Berkowitz, T. Darden, H. Lee and L. G. Pedersen, *J. Chem. Phys.*, 1995, **103**, 8577–8593
- (S24) D. Van Der Spoel, E. Lindahl, B. Hess, G. Groenhof, A. E. Mark and H. J. C. Berendsen, *J.*

Comput. Chem., 2005, **26**, 1701–1718.

- (S25) W. Humphrey, A. Dalke and K. Schulten, *J. Mol. Graph.*, 1996, **14**, 33–38.
- (S26) M. Montal and P. Mueller, *Proc. Natl. Acad. Sci. U. S. A.*, 1972, **69**, 3561–3566.
- (S27) B. Hille, *Ionic channels of excitable membranes*, Sinauer Associates, Sunderland, MA, 1992.
- (S28) D. S. D’Astolfo, R. J. Pagliero, A. Pras, W. R. Karthaus, H. Clevers, V. Prasad, R. J. Lebbink, H. Rehmann and N. Geijsen, *Cell*, 2015, **161**, 674–690.
- (S29) F. Yokoya, N. Imamoto, T. Tachibana and Y. Yoneda, *Mol. Biol. Cell*, 1999, **10**, 1119–31.
- (S30) C. Savino, L. Federici, R. Ippoliti, E. Lendaro and D. Tsernoglou, *FEBS Lett.*, 2000, **470**, 239–243.
- (S31) F. Guo, D. N. Gopaul and G. D. van Duyne, *Nature*, 1997, **389**, 40–46.
- (S32) E. O. Saphire, P. W. Parren, R. Pantophlet, M. B. Zwick, G. M. Morris, P. M. Rudd, R. A. Dwek, R. L. Stanfield, D. R. Burton and I. A. Wilson, *Science*, 2001, **293**, 1155–1159.
- (S33) H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov and P. E. Bourne, *Nucleic Acids Res.*, 2000, **28**, 235–242.
- (S34) E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng and T. E. Ferrin, *J. Comput. Chem.*, 2004, **25**, 1605–1612.
- (S35) P. Capasso, M. Aliprandi, G. Ossolengo, F. Edenhofer and A. de Marco, *BMC Biotechnol.*, 2009, **9**, 80.
- (S36) J. K. Armstrong, R. B. Wenby, H. J. Meiselman and T. C. Fisher, *Biophys. J.*, 2004, **87**, 4259–4270.
- (S37) B. Bjellqvist, G. J. Hughes, C. Pasquali, N. Paquet, F. Ravier, J. C. Sanchez, S. Frutiger and D. Hochstrasser, *Electrophoresis*, 1993, **14**, 1023–1031.