Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information for

Immobilization of membrane proteins on solid supports using functionalized β-sheet peptides and click chemistry

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1. Materials and methods

1.1 General materials

The chemicals were obtained from Sigma-Aldrich unless otherwise indicated. The cDNA sequence was confirmed by DNA sequencing services at the University of Alberta's Molecular Biology Service Unit.

1.2 Synthesis and characterization of FBP1s

Fmoc-L-octylglycine ((octyl)Gly) and functional amino acids, including Fmoc-L-propargylglycine for pBP1, and N_{α}-Fmoc-3-azido-L-alanine for aBP1, were all purchased from AAPPTec. An automated solid-phase peptide synthesizer (Focus XC or Infinity 2400 (AAPPTec)) was used to prepare the BP sequences following standard batchwise Fmoc chemistry methodology. Specifically, 20% pipiridine in DMF was used for Fmoc deprotection. Rink amide 4-methylbenzhydrylamine resin (AAPPTec), with a substitution of 0.52 mmol/g was chosen as the solid support to load the amino acids. For amino acid coupling, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and N-methylmorpholine (NMM) were used as the coupling reagents. In the case of aBP1, the coupling of N_{α}-Fmoc-3-azido-L-alanine was performed using (benzotriazol-1-yl-oxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (AAPPTec) instead of HCTU. After the last cycle of deprotection, the peptides were acetylated at the N-terminus. The product was then mixed with a cleavage cocktail consisting of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% DI water for 1.5 h with shaking to cleave the peptides off from their support resins. The resins were removed by filtration. The peptides were then precipitated in 50 ml of cold ether, centrifuged at 9000 rpms for 8 minutes. The

supernatant was discarded. The raw peptide was re-dissolved in a 1:1 acetonitrile:H_2O solution and lyophilized (Labconco FreeZone 4.5 plus). Purification of all peptides was achieved through reverse-phase HPLC (AAPPTec UV3000 detector at 220 nm) using a Spirit Peptide 120 C18 column (10 µm, 25×2.12, AAPPTec) and using acetonitrile-H_2O, 0.1% TFA as the mobile phase. The subsequent pure product was then lyophilized once more. The molecular weights of BPs were confirmed by ESI-MS (Agilent 1946 LC/MS, Agilent Technologies).

1.3 Preparation of AqpZ, NaChBac, ChIEF-mCitrine and F_oF_1-ATPase

Plasmid pTrc10HisAq[1] was used as a template to clone the AqpZ coding gene, which is subsequently inserted between the NdeI and HindIII sites of a pET28a-Novagen vector (EMD Millipore). The codon encoding Arg3 was changed from *aga* to *cgt* in order to increase the translation efficiency. The resulting pET28a-6×His-AqpZ was used for overexpression in *E. coli* BL21 (DE3). A fresh colony was grown in Luria broth (LB) with 30 µg/ml of kanamycin for 13-16 h at 37 ˚C. The cell culture was diluted 100× into a Terrific Broth (TB) supplemented with 0.02 M of glucose and 15 µg/mL of kanamycin. The cells were allowed to grow at 37 ˚C until the optical density at 600 nm (OD600) reached 1.0. Protein overexpression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) with a final concentration of 0.5 mM. Cells were allowed to grow for another 4 h at 37 ˚C. The cells were harvested by centrifugation at 9000 g for 15 minutes and the pellets were frozen at -20 ˚C. The protein purification was carried out as described[1] except that the DDM

concentration in the washing and elution buffer was reduced to 0.05% to facilitate the detergent's removal in downstream experiments.

The cDNA coding for NaChBac was cloned from a NaChBac expressing *E. coli* strain[2] and inserted into a pET28a vector (Novagen) between the NdeI and EcoRI restriction sites. As a result, a hexahistidine tag was installed to the N-terminus of NaChBac and was linked by a 10 amino acid spacer (Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His). The overexpression and the purification were performed by following similar procedures to that of AqpZ.

ChIEF-mCitrine was overexpressed in *Pichia pastoris* and purified by IMAC as previously described.[3] F_oF_1-ATPase was extracted from thermophilic bacterium PS3 and purified by ion exchange chromatography following previously published protocol.[4] All purified proteins were confirmed by SDS-PAGE and quantified using Direct Detect® (MilliporeSigma), an infrared (IR)-based protein quantification instrument.

1.4 Preparation and characterization of the protein:peptide complexes

To prepare the protein:peptide complexes the purified protein was mixed with the corresponding β-sheet peptide at 1:50 molar ratio. The mixture was incubated at 4 °C for at least 1 h. Subsequently, the detergent was removed by dialysis at 4 °C. The dialysis buffer was supplemented with Bio-Beads™ SM-2 resin (Bio-Rad) at a final concentration of 20 mg per 100 ml buffer and changed 3 or 4 times over a 3-day period. Protein concentration and residue detergent concentration was measured using Direct Detect®. Particularly, the DDM removal was monitored by recording the IR absorbance of the control samples (i.e. peptide-free protein) at

2856 cm^{-1} which corresponds to the C-H symmetric stretching mode in DDM and quantified using a house-made calibration curve.

To obtain the far-UV circular dichroism (CD) spectra, the purified protein or the protein:peptide complex was buffer exchanged into 10 mM NaH_2PO_4 (pH 7.4, supplemented with 0.05% DDM in the case of protein/DDM samples) using a centrifugal device (MilliporeSigma) immediately before the measurement. A quartz cuvette with a 0.1 cm path length was used for the measurement. A Jasco J-810 CD Spectrometer (Easton, MD, USA) was used with the following settings: scanning wavelength 185 nm to 260 nm, 1.0 nm step, 1.0 nm bandwidth, and a 3 second collection time per step at 25 °C. The spectra were smoothed using the Savitzky-Golay function supplied in the software.

The Direct Detect® was used for the Fourier transform infrared (FTIR) spectra collection. 2 µL of the sample was spotted on a hydrophilic polytetrafluoroethylene (PTFE) membrane that is commercially available from MilliporeSigma. The membrane is then heat-dry and analyzed. The reported spectra have been corrected using the corresponding sample buffer as blank.

The enzyme activity of F_oF_1-ATPase was quantified by its ATP hydrolysis activity using the PiColorLock™ ATPase assay kit (Innova Biosciences) following the manufacturer's protocol. Specifically, the inorganic phosphate (P_i) generated by the ATP hydrolysis was quantified by a colorimetric reaction between P_i and a malachite green reagent provided in the kit. A calibration curve is created using standard P_i with concentrations ranging from 2.5 to 100 µM. The purified F_oF_1-ATPase or the protein:peptide complex was buffer exchanged into an ATP-free

assay buffer (50 mM Tris-SO_4, 5 mM p-aminobenzamidine, 40 mM ε-amino-N-caproic acid, 0.5 mM 1,4-dithiothreitol, pH 8.0) immediately before the enzyme assay. Subsequently, ATP (final concentration 1 mM) was added and the hydrolysis was allowed to proceed for 20 min at 42 °C. The background signal caused by the enzyme in the absence of ATP and the non-enzymatic ATP hydrolysis was corrected.

To test the propargyl functionality in pBP1 after it binds to the IMP, the Click-IT TAMRA protein analysis detection kit (Invitrogen) was used. The F_0F_1-ATPase:pBP1 complex and F_0F_1-ATPase/DDM were labeled following the procedures in the manufacturer's manual. Excess dye or reaction mixture was removed using a HiTrap 5 mL desalting column (GE Healthcare). Fluorescence spectra were acquired on a QuantaMaster spectrofluorometer (Photon Technology International) with an excitation at 480 nm to collect the full emission peak of TAMRA. Excitation maximum of TAMRA is 545 nm.

To determine the hydrolysis activity of the immobilized F_0F_1-ATPase:aBP1, 500 µL of the F_0F_1-ATPase:aBP1 and the F_0F_1-ATPase:BP1 were first incubated with 40 µL of the dibenzocyclooctyne- (DBCO-) agarose (Jena Bioscience, Germany), respectively, with gentle shaking at room temperature for 2 hr. The products were washed three times with the ATP-free assay buffer by centrifuging the agarose to the bottom (3000 ×g, 4°C, 1 min) and removing the supernatant. The washed products were then resuspened with 500 µL of assay buffer and used for ATP hydrolysis assay as aforementioned. The mixtures were frequently shaken during the incubation to prevent the sinking of the agarose to the tube bottom. After the

hydrolysis reaction, the supernatant was removed and test for the presence of P_i. The F-ATPase:peptide agarose beads were washed using the assay buffer for 4-6 times, resuspended with the original volume (500 µL) of assay buffer, stored at 4°C, and used for ATPase activity assay again on the second day. The same washing procedures and activity assay were performed for a third time on the third day.

1.5 Immobilization of the IMP:FBP1

To prepare FITC-AqpZ, purified AqpZ in PBS/DDM buffer was reacted with fluorescein-5-Isothiocyanate (FITC, Invitrogen) at a molar ratio of 1:10 at room temperature for 1 h. The excess amount of FITC was removed using a 5 mL HiTrap desalting column (GE Healthcare). The average labeling efficiency was calculated to be 1-2 FITC per protein monomer, depending on the batches.

The alkyne-functionalized PEG-coated glass coverslip was purchased from MicroSurfaces (Englewood, NJ, USA). The FITC-AqpZ:peptide complex was mixed with 0.03 eq. $CuSO_4$, 0.2 eq. sodium ascorbate, 0.03 eq pentamethyl-diethylenetriamine, and glycerol (final concentration 10%). Glycerol was added to prevent the spread of the drops when added to the surface of the glass coverslip. Immediately after the mixing, 0.5 µL of the mixture was spotted on the glass coverslip and incubated in a humidified box for 1 h. The excess protein:peptide complex was collected and loaded on the SDS-PAGE under pseudo-native condition (i.e. sample was not heated) to find out the oligomeric status of the complexes. The excess sample and reagent on the coverslip was then washed away using PBS supplemented with 0.05% Tween-20. Fluorescent imaging was

taken using the ImageQuant LAS4000 gel imager (GE Healthcare) using a FITC filter set. Quantification of the fluorescence intensity was performed using ImageJ. Immobilization of the FITC-AqpZ:pBP1 on the azido-functionalized polysulfone membrane was carried out using similar click reaction conditions. The preparation of the azido -functionalized polysulfone will be described in a separated work.[5]

To determine the immobilization efficiency, the alkyne-functionalized coverslip (22 mm × 22 mm) was incubated with 300 µl of FITC-AqpZ:aBP1 on a rocking platform. The aforementioned click reaction condition and rinsing procedure were used. 2 µL of FITC-AqpZ:BP1 prepared in parallel and with known concentration was spotted next to the coverslip at the fluorescent imaging step to create the calibration curve for the protein amount quantification. The fluorescent intensity, spot size and the integrated fluorescent intensity were analyzed using ImageJ.

1.6 Molecular modeling and docking of the β-sheet peptides

The BP1 (acetyl-(octyl)Gly-Ser-(met)Leu-Ser-Leu-Asp-(octyl)Gly-Asp-NH_2), pBP1 (acetyl-(azido)Ala-(octyl)Gly-Ser-(met)Leu-Ser-Leu-Asp-(octyl)Gly-Asp-NH_2) and aBP1 (acetyl-(propargyl)Ala-(octyl)Gly-Ser-(met)Leu-Ser-Leu-Asp-(octyl)Gly-Asp-NH_2) peptide structures were built using the MOE software.[6] The four non-standard residues, namely (octyl)Gly, (met)Leu, (azido)Ala, and (propargyl)Ala, were introduced to the system using the Pyred server.[7] β sheet dimer initial structures were prepared by docking two monomers using Zdock[8] and MOE software. The structures were minimized using Amber software.[9] β-sheet

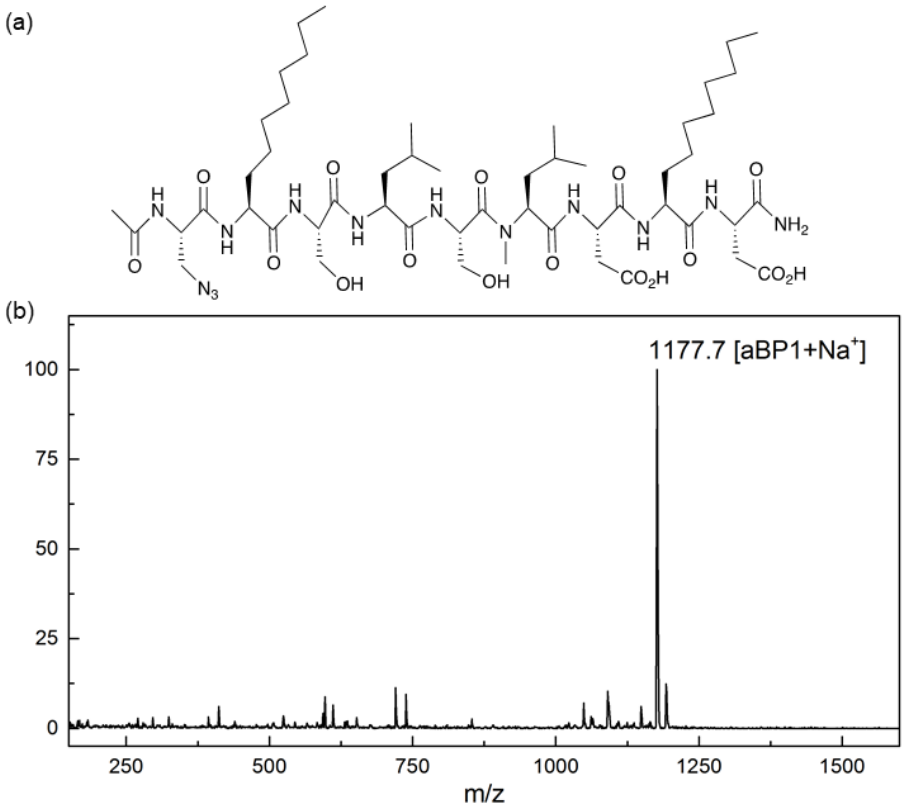
formation is observed during the further all atom replica exchange molecular dynamics simulations.

2. Supplementary table

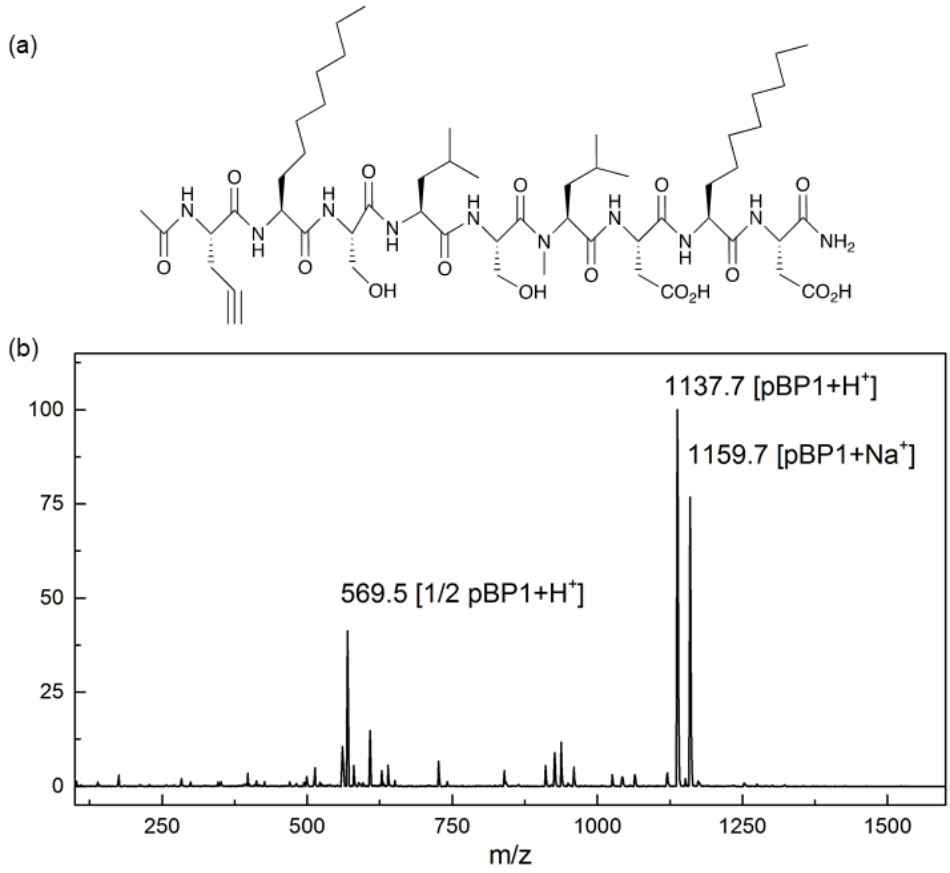
Supplementary Table S1. Peak and valley positions of the CD spectra for the membrane proteins and their peptide complexes. NA: not applicable. ND: not determined. Diff.: difference spectra.

		Wavelength (nm)						
		DDM	BP1	aBP1	pBP1	diff. (BP1)	diff. (aBP1)	diff. (pBP1)
AqpZ	Peak 1	196	193	191	191	NA	NA	NA
	Valley 1	210	210	208	207	203	205	205
	Valley 2	223	223	222	222	220	219	220
NaChBac	Peak 1	194	191	189	ND	NA	NA	NA
	Valley 1	209	209	208	209	200	200	198
	Valley 2	220	220	222	223	222	222	226
ChIEF-mCitrine	Peak 1	194	191	193	190	NA	NA	NA
	Valley 1	208	208	209	208	210	210	210
	Valley 2	224	221	221	220	220	220	220
F_0F_1-ATPase	Peak 1	192	192	192	192	NA	NA	NA
	Valley 1	209	209	208	208	205	205	205
	Valley 2	224	223	223	226	224	226	226

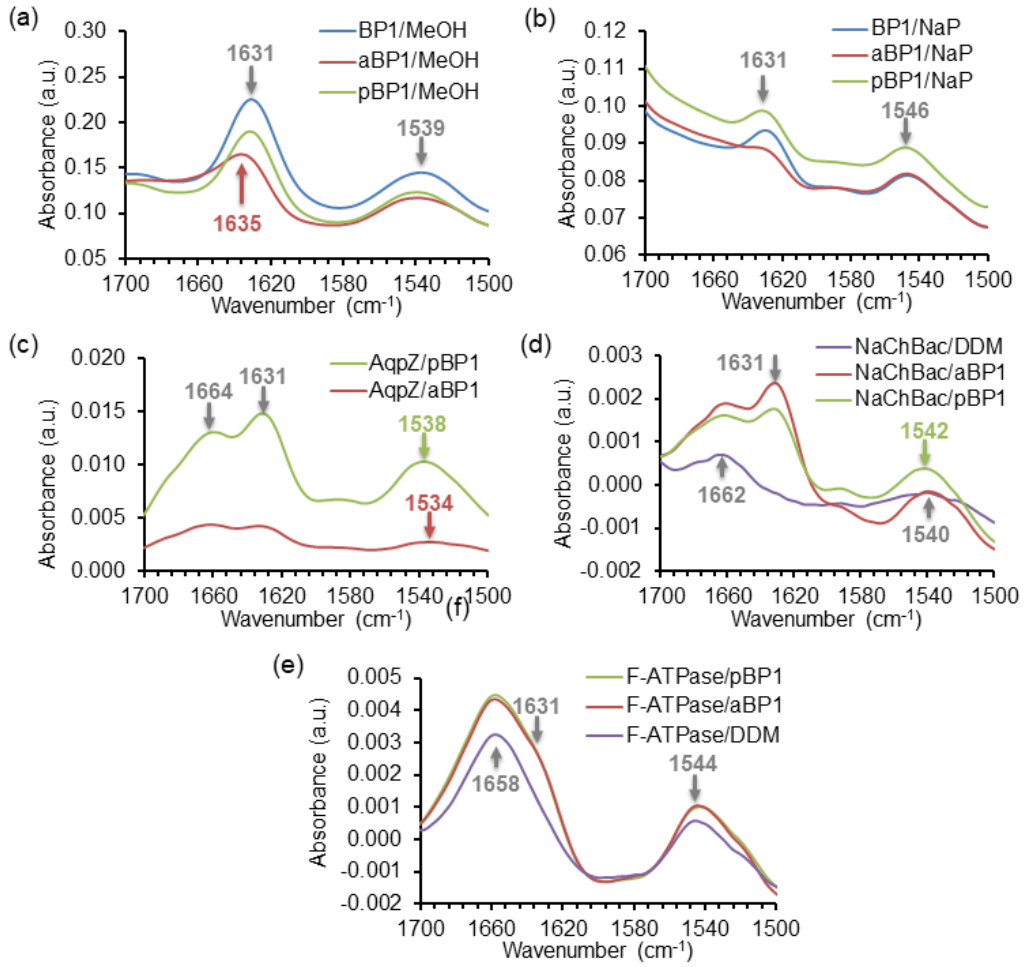
3. Supplementary figures



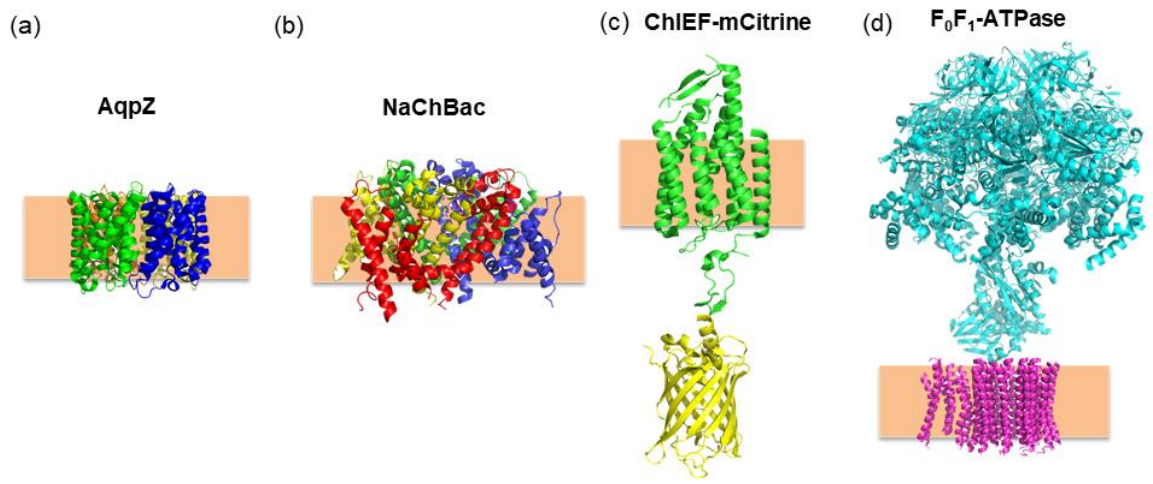
Supplementary Figure S1: Chemical structure (a) and ESI-MS spectrum (b) of azido-BP1.



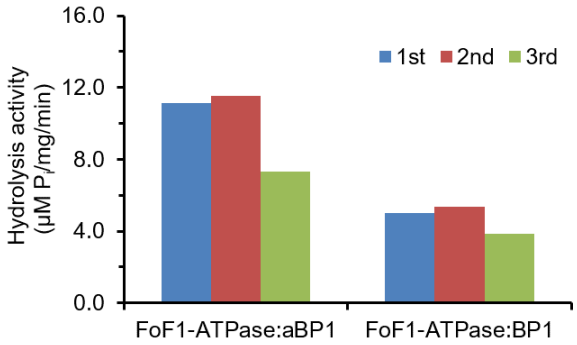
Supplementary Figure S2: Chemical structure (a) and ESI-MS spectrum (b) of propargyl-BP1.



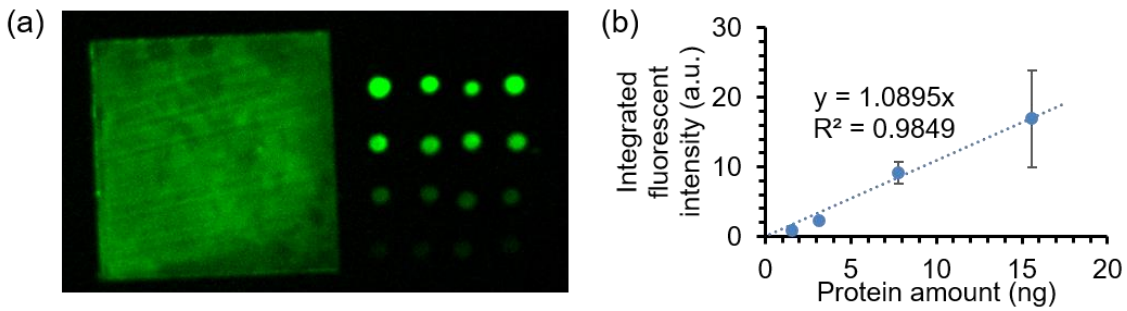
Supplementary Figure S3: FTIR spectra of the β-sheet peptides and the IMP:peptide complexes. The presence of a major peak around 1630 cm^{-1} in the Amide I (1600-1690 cm^{-1}) band indicates the formation of β-sheet structure in the peptides within the IMP:peptide complexes.[10] The presence of the major peak around 1660 cm^{-1} is the result of the α-helix-rich structure in the IMPs. [10] A peak in the Amide II (1480-1575 cm^{-1}) region is also found for all samples.



Supplementary Figure S4: Cartoon representation of the IMPs' structures that were used in this work. The transmembrane region is indicated by the brown shade. (a) The tetrameric AqpZ structure generated using PDB file 2ABM.[11] (b) The tetrameric NaChBac structure illustrated using its homologue Na_vRh (PDB ID 4DXW).[12] (c) Structure illustration of ChIEF-mCitrine generated by assembling the structures of C1C2 (a ChIEF variant, PDB ID 3UG9)[13] and Citrine (PDB ID 3DPW)[14]. (d) Structure illustration of F_0F_1-ATPase generated by assembling the structures of its transmembrane F_0 domain (PDB ID 1C17)[15] and its cytosolic F_1 domain (PDB ID 1E79)[16].



Supplementary Figure S5: Apparent enzyme activity of the F_0F_1-ATPase:FBP1 immobilized on the DBCO-agarose beads. Blue: measured right after immobilization; red: the same samples were washed and measured on the second day; green: measured on the third day. The reduced apparent enzyme activity for F_0F_1-ATPase:aBP1 on the third day was largely due to the lost of the agarose beads during repeated washing. While substantial non-specific binding was observed for the DBCO-agarose, the data shows the stability of both of the F_0F_1-ATPase:BP1 and F_0F_1-ATPase:aBP1 under repeated washing and over three days.



Supplementary Figure S6: Immobilization efficiency of the FITC-AqpZ:aBP1 on the alkyne-functionalized coverslip. (a) Fluorescent image of the immobilized FITC-AqpZ:aBP1 (left) and 2 uL-spots of the FITC-AqpZ:aBP1 at varied concentrations (right). (b) Calibration curve used for determination of the immobilization density of the FITC-AqpZ:aBP.

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