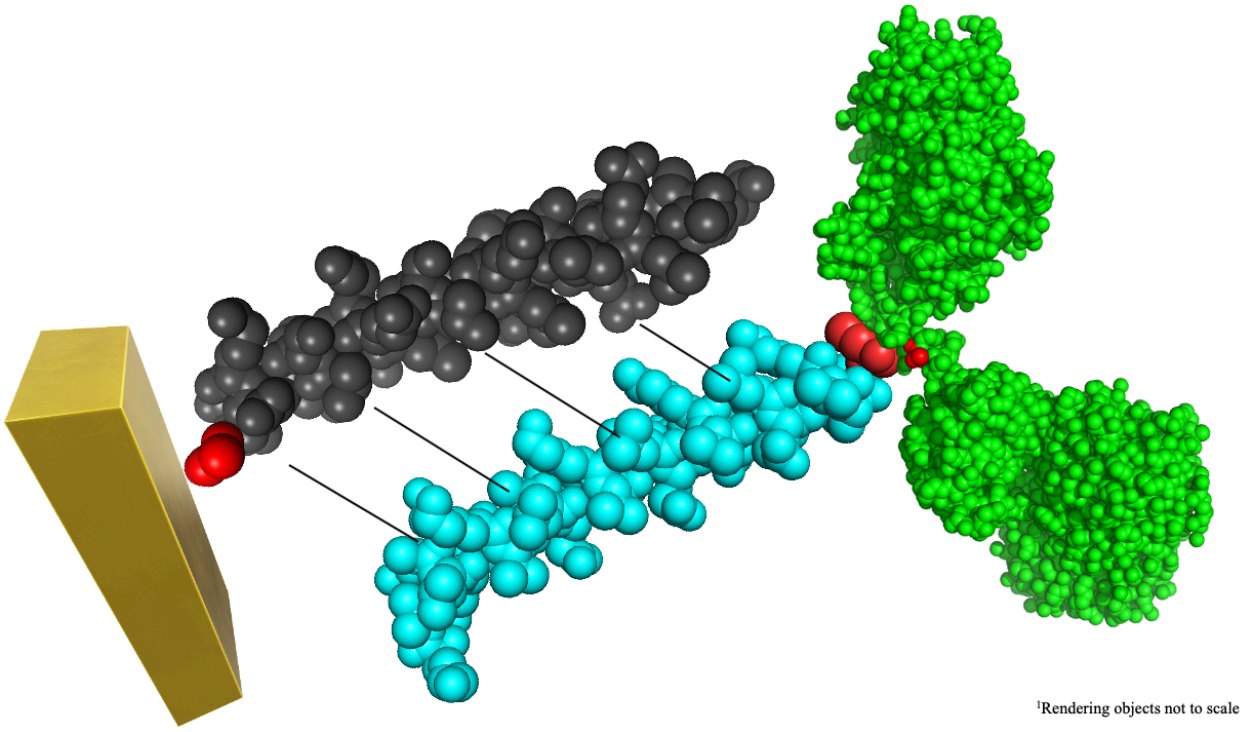
Practical guide for development of a metabolite biosensor using immobilized fluorescent proteins.

Sono Khan[1,2], Genevieve Norris-Roozmon[1,3], Kody Klupt[1,2], Byron Hunter[1,2], Nolan Neville[1,2]

[1]Queen's University at Kingston [2]Department of Biomedical and Molecular Sciences and [3]Department of Chemical Engineering

A *Queens Genetic Engineered Machine Team* project for the *iGEM 2020 competition*.

More project information can be found at queensigem.ca



[1]Rendering objects not to scale

Project overview and purpose.

This year's project goal is to produce a novel transdermal metabolite biosensor for point-of-care diagnostic quantification of phosphate, potassium, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23) and glucose levels in sweat or interstitial fluid. We will generate a genetically encoded Förster resonance energy transfer (FRET) metabolite sensor by fusing existing binding proteins to fluorescent proteins (FPs). To quantify physiologically relevant levels of metabolites, highly optimized mNeonGreen and mCherry fluorophore pairs will be used as these are relatively stable and impose a large fluorescent linear range as they are quite intense (bright) (1). Upon conjugation of fluorophores, we will use site-directed mutagenesis to add a Cysteine residue to the protein. This will serve as a critical first step to immobilizing the biosensor onto a microfluidic surface. Upon purification of the recombinant protein, we aim to integrate our metabolite biosensor into a microfluidics system that will exist in a handheld device of our own design. Through this, we will measure the fluorescence signal given off by the protein construct and correlate it to a metabolite concentration (1). This part of the project will make use of open source software based in MATLAB, and open source hardware that has previously been turned into a spectrometer.

Our research will involve many techniques based in both the wet and dry lab. The wet lab methods will include plasmid design, site-directed mutagenesis, DNA cloning and restriction digestion, polymerase chain reaction, bacterial transfection, bacterial protein expression, protein purification, immunoblotting, and fluorescent microscopy. Dry lab techniques will revolve around prototype design and molecular modelling, thereby involving CAD, PyMol, MATLAB coding, website design, and 3D printing. Both dry lab and wet lab techniques will then be used in conjunction to purpose a microfluidics system for biosensing.

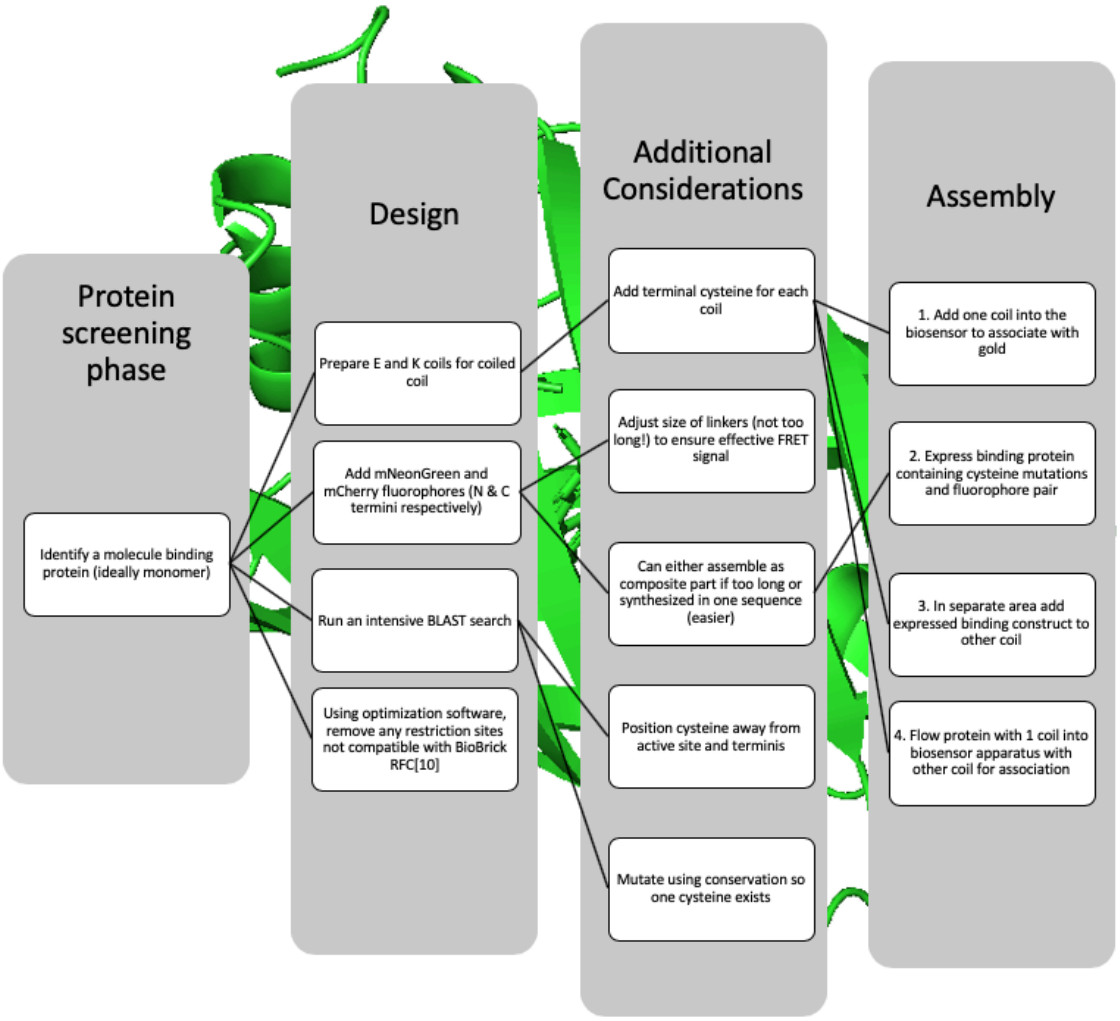
The scope of our project is immense in the Chronic Kidney Disease (CKD) landscape. Phosphate levels are associated with a slew of cardiovascular and renal complications, with hyperphosphatemia serving as a direct stimulus to vascular calcification – a leading cause of the morbidity and mortality associated with CKD (2). Currently, there is no point-of-care method for phosphate detection, with blood analysis being the main clinical diagnostic tool. This method is slow, and insufficient to measure the fluctuating levels of phosphate in a CKD patient (3).

The limits of clinical phosphate detection were further discussed in a meeting with Dr. Rachel Holden (Division of Nephrology, Department of Medicine) and Dr. Michael Adams (former Head of the Department of Biomedical and Molecular Sciences) who spoke to the numerous limitations of blood testing for phosphate detection while strongly supporting our project and the significance it can have in the clinic. According to Dr. Holden, phosphate levels of CKD patients are taken approximately once every 6 weeks, which provide little insight into the state of CKD. She discussed a need for real-time and fast detection of phosphate levels, which is what our project aims to provide. By giving physicians and patients the ability to measure their own phosphate levels quickly, accurately, and continuously, our project can truly make a difference in the lives of CKD patients around the world.

Our research will be conducted under Dr. Allingham (PI), and in Dr. Capiciotti's lab from the Department of Biomedical and Molecular Sciences. We will be advised by the previously mentioned professors, along with Dr. Adams, Dr. Holden, Dr. Davies, and graduate students Byron

Hunter and Nolan Neville. However, the QGEM team will be conducting the research alone, and we are undergraduate students. Each year QGEM and the iGEM competition provide undergraduate students with an invaluable opportunity to learn about synthetic biology techniques, help solve real-world problems, and ultimately represent Queen's University at the international level.

Schematic overview of protein construct design.



Overview of protein construct design and assembly process. To develop the metabolite biosensor using an immobilized fluorescent binding protein, with cysteine modifications, and the coiled coil system – a schematic overview is created. Process should be followed Left-Right from Protein screening phase to Assembly, respectively. Branching indicates subsequent steps.

It is important to consider protein screening, design, and assembly when making the protein constructs for the biosensor device. This schematic overview serves as a representation to how proteins for metabolite detection are selected and modified to work in a biosensor. Asides from the protein construct with the FRET pair, the immobilization technique requires a coiled coil system, cysteine modifications, and if possible – modelling is recommended such as a molecular

dynamics simulation (4). The protein construct design will be looked at in further detail in subsequent sections.

Protein screening.

It is very important to ask yourself why I am adding this specific metabolite binding protein before attempting to build and model a construct. While biosensors can be created to detect metabolite levels in typical blood serum, there is an increasing demand by patients and clinicians for detection of metabolites using non-invasive measures such as sweat or interstitial fluid collection, as evidenced by our group meeting with these audiences. Therefore, if a metabolite for detection comes to mind, we recommend performing a literature search to see if there is an existing correlation between blood serum or sweat/interstitial fluid levels. Ask if there is no correlation described in literature, has there been evidence that different levels of sweat/interstitial fluid of this metabolite are associated with disease? This step is crucial – should no relationship or such studies exist, this will prolong the time for testing the device. We are not saying do not attempt this but exert caution that testing physiological sweat/interstitial fluid levels may be expensive and will take a long time.

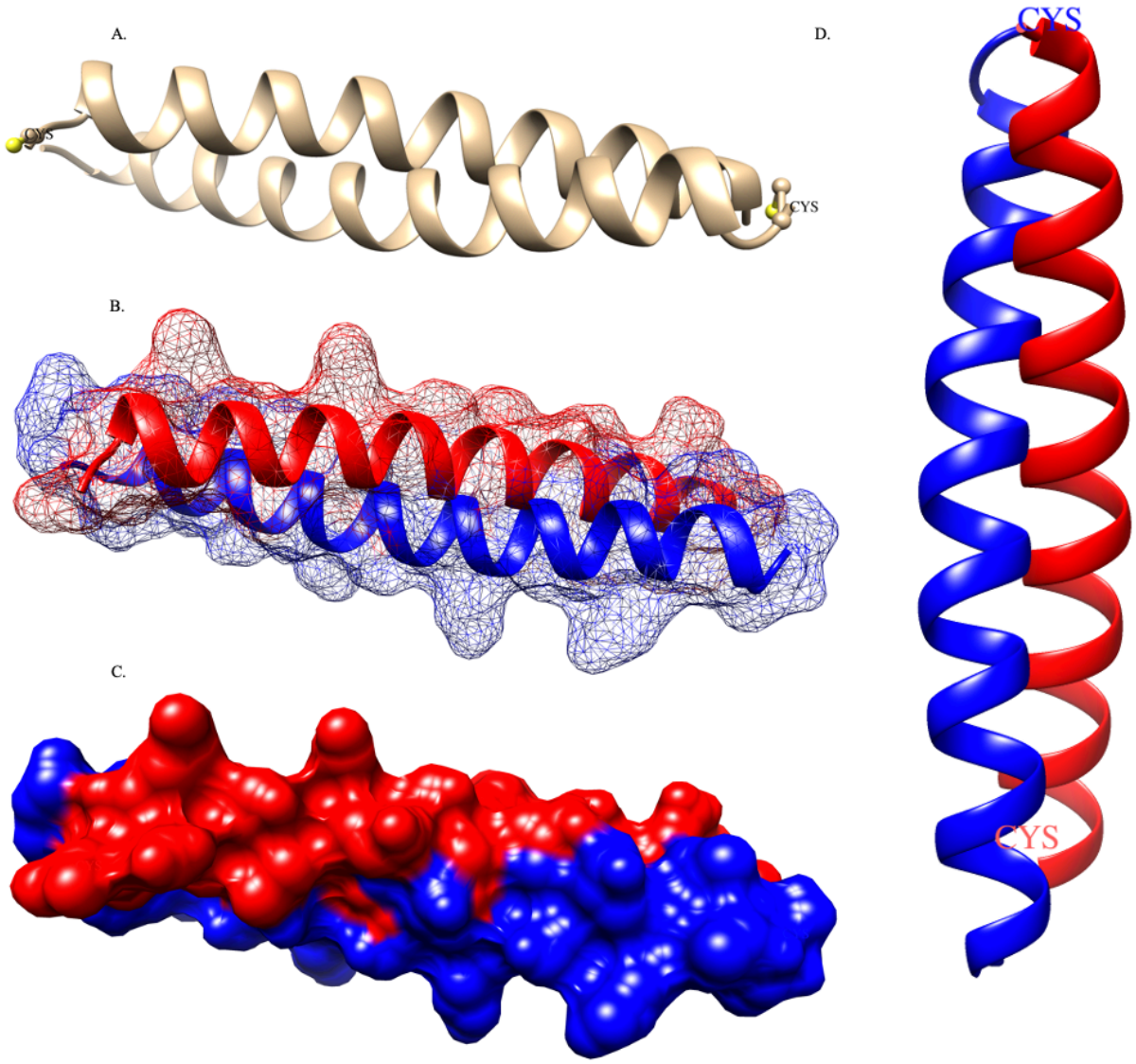
Now presuming you have found an appropriate metabolite you would like to measure; it is important to find a protein that will bind to it. Our group has seen evidence of bacterial proteins that bind metabolites such as phosphate binding protein or potassium binding protein (5,6). These are typically cytosolic proteins that are often small, under about 30 kD in size (5–9). It would be wise to avoid membrane proteins as these are still difficult to express with existing technology and may be considerably problematic to incorporate into our biosensor. Another consideration to make is the active sites of these proteins binding specific metabolites. For example, we found in literature it was important to mutate the active sites of Troponin C to selectively bind calcium ions and avoid magnesium competitive inhibition (7). These adjustments are not very difficult but are an important consideration early on in the design phase. [Note: Troponin C was later removed from our project for clinical reasons.]

All the protein structures including our fluorophores were obtained from the Protein Data Bank (10). Here you can see structure and sequence of the protein. Since this technique uses cysteines to immobilize the protein, should your protein have cysteines, check to see if these form a disulfide bond and how many there are (more on this later). Should there be disulfide bonds which would need to be removed, this may be problematic as the protein structure may be significantly affected, subsequently affecting function. Further considerations would include looking to see if the protein has multiple subunits. All of our proteins used are exclusively monomers. This makes it easier for us to control fluorophore binding to the terminal regions and any cysteine mutations. Our last point of consideration is termini structure. Are the proteins two N and C termini's right next to each other? If so, this might be problematic for fluorescent signaling. Look for a protein that will have an N and C termini reasonably separated. A lot of discretion and patience is needed for the protein screening phase, but should these considerations described be met, you and your team will save time, money, and the agony of having to go back to the drawing board.

Design considerations.

With the initial protein screening phase completed you can begin making designs changes to your protein of interest if required. Our initial advice is to run an intensive BLAST search for protein homology to assist you in making conservative substitutions should they be necessary, especially for removal of cysteine residues (11). Our group used Phyre2 to do this, a web-based protein homology application that only requires you to insert a proteins amino acid sequence (12). We couldn't recommend this enough; the homology report was visually helpful and included in some instances hundreds of similar proteins (they aren't paying us to say this).

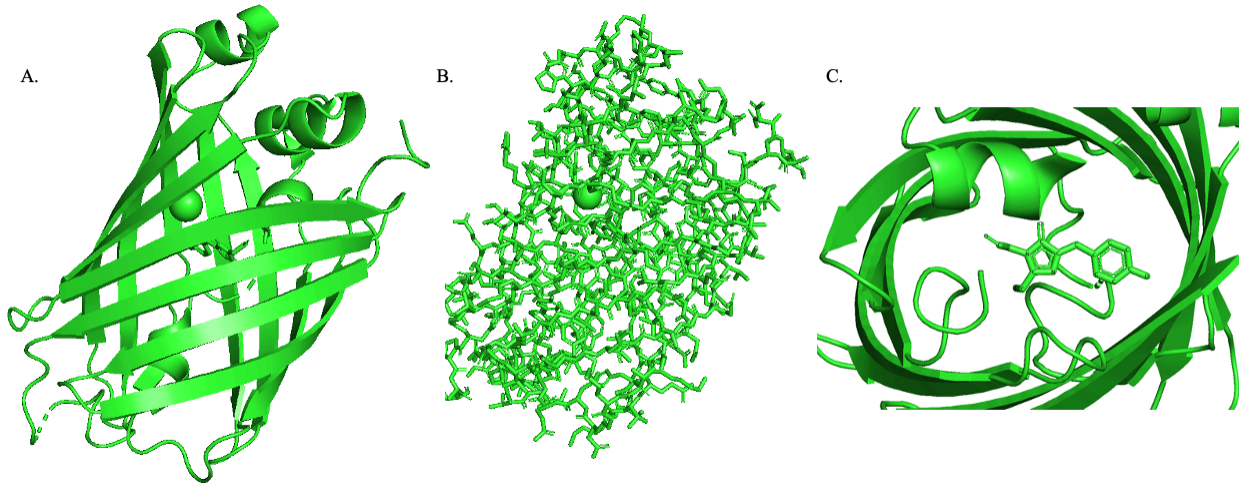
It is now important to create your E and K coils to form a coiled coil structure that is used for protein immobilization. The E coil alpha helix is made of five-heptad repeats which work in conjunction with the K coil alpha helices five-heptad repeats (4). The E heptad and K heptad contain sequences of EVSALEK and KVSALKE, respectively, where the hydrophobic residues leucine and valine form a hydrophobic interface, stabilizing the coiled coil heterodimeric structure (4). This hydrophobic effect is further amplified by repetition of the heptad repeat and provides great stability (4). Glutamate and lysine at the outer positions of the coils form electrostatic interactions further stabilizing the heterodimer structure (4). Addition of a cysteine to the C-termini of either alpha helix provides a method of protein immobilization. One coils cysteine may bind a surface such as gold on the inside of a biosensor, while another coils cysteine can bind a proteins cysteine residue. The alpha helices may then associate, and a protein can therefore be immobilized. This method of protein immobilization is cost effective and simple compared to existing biochemical techniques. Note that these coils are expressed separately from one another and the binding protein construct. Their nucleotide sequences will therefore be ordered separately.



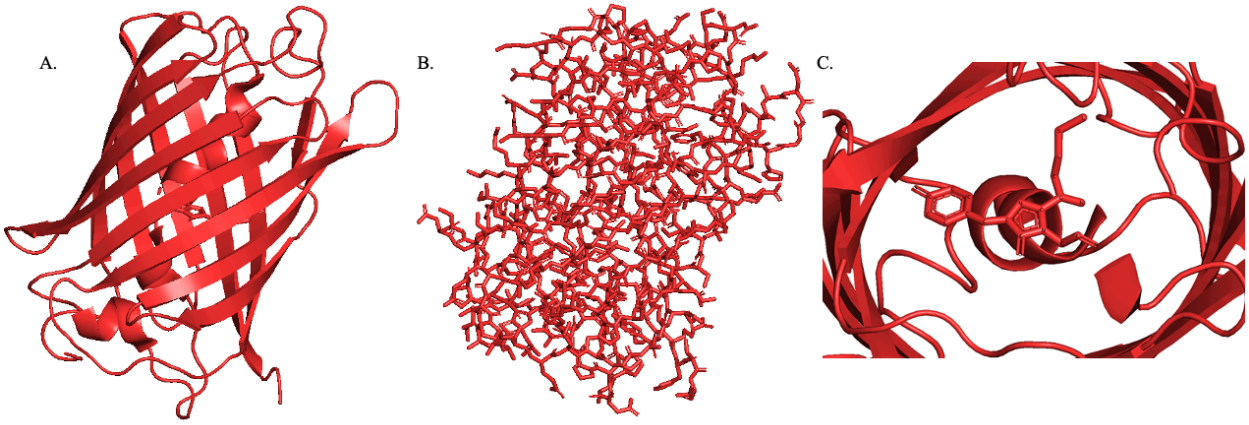
E/K coiled coil system. All protein residues are shown with the E and K coils in red and blue (B-D) , respectively, using Chimera software. E and K coils each consist of five-heptad repeats with a hydrophobic core stabilized by glutamates and lysine residues surround the interface. A. Coil's in a cartoon preset with each coil's cysteine labelled and displayed in a ball-and-stick preset. Note the sulfur atom in yellow. B. Coiled coil system in a mesh preset to visualize volume in 3D space. C. Volume in 3D space further enhanced with a solid surface preset, further, highlighting the interwoven nature of the coiled coil. D. Standard cartoon preset with the coiled coil alpha-helices oriented vertically.

Additionally, you must add your fluorophore pairs to the binding protein. We use a mNeonGreen and mCherry pair on the N and C termini's, respectively, for our proteins. This pair is bright, stable, and has an extended dynamic linear range over a simpler GFP-RFP pairing (1). Linker size is important to prevent steric clash with the binding protein – however, consider if linkers are too long which may impede fluorophore association (13). Using a protein visualization and design software (we have had a good experience with Chimera), you may get a better understanding as to whether steric clash exists and how long the termini linkers should be to avoid this (14). With the binding proteins we have used, never have our linkers exceeded more than a handful of amino acids in length (5–9). It is also important to generate a full construct of binding protein with

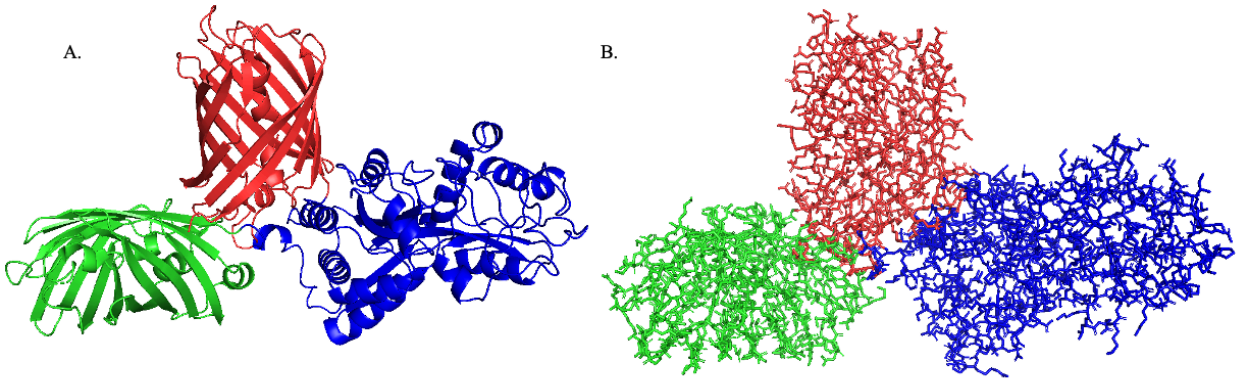
fluorophores for when you order your synthetic DNA to be expressed. Do note that many companies will not exceed a certain length such as 2500 nucleotides so a further assembly method may be required, and the protein construct will need to be ordered in parts.



mNeonGreen fluorescent protein used for attachment to N-termini of binding proteins. Protein structures were obtained from the RCSB Protein Data Bank. All protein images were created using PyMOL. A. mNeonGreen protein in a cartoon preset displaying a fluorescent proteins characteristic beta barrel structure. B. A stick preset of mNeonGreen, insightful into the fluorescent proteins volume in 3D space. C. A sliced image of mNeonGreen exposing the chromophore center, vital for fluorescence (atoms are labelled by element).

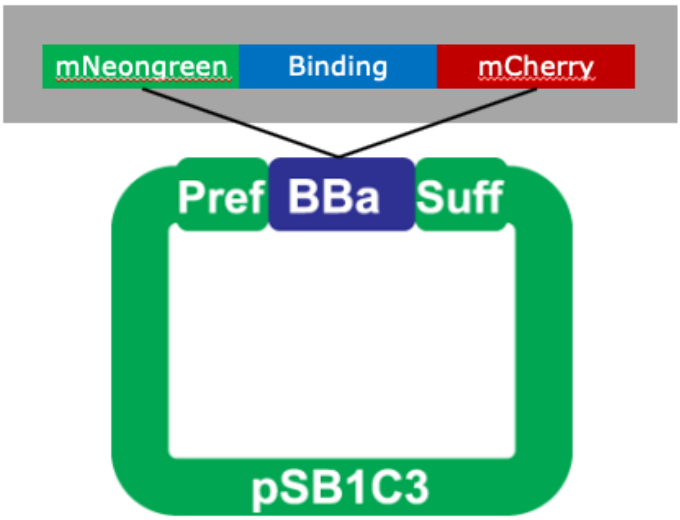


mCherry fluorescent protein used for attachment to C-termini of binding proteins. Protein structures were obtained from the RCSB Protein Data Bank. All protein images were created using PyMOL. A. mCherry protein in a cartoon preset displaying a fluorescent proteins characteristic beta barrel structure. B. A stick preset of mCherry, insightful into the fluorescent proteins volume in 3D space. C. A sliced image of mCherry exposing the chromophore center, vital for fluorescence (atoms are labelled by element).



Phosphate binding protein fluorescent construct. Protein structures were obtained from the RCSB Protein Data Bank and a construct was made using Chimera software. Torsion angles between fluorescent and binding proteins are adjusted for display purposes. mNeonGreen (green), binding protein (blue), and mCherry (red) are all displayed using PyMOL. A. Phosphate binding protein with a cartoon preset coupled with two fluorophores (mNeonGreen and mCherry). B. Construct in a stick preset to provide insights regarding the construct volume in 3D space.

Another design consideration you must make is what plasmid you will use and optimizing your DNA to avoid the restriction sites you want to use, to remove your DNA from a plasmid. All of our proteins are compatible with BioBrick RFC [10] standards and use the pSB1C3 plasmid for shipment (15–17). Codon optimization may need to occur to meet these requirements and can be done using a variety of software applications. The expression plasmid of choice is the pET24D plasmid, decided in consultation with our advisors. A His tag can be included on either termini of the protein to aid in the purification steps.

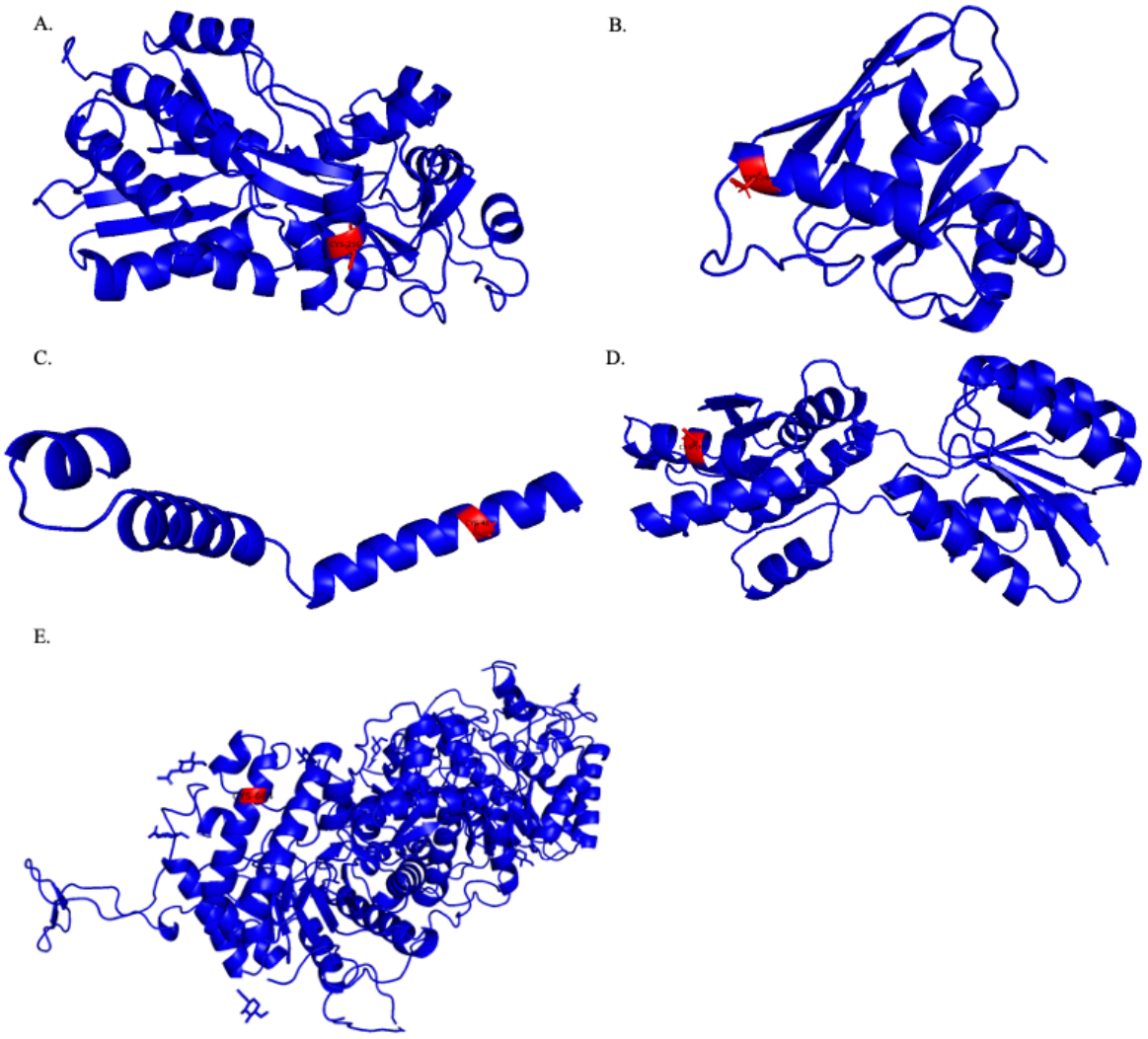


Plasmid design. Using the BioBrick RFC [10] compatibility standards a generic construct can be created where BBa is the protein construct inserted into the pSB1C3 plasmid.

Cysteine mutations and limitations.

The most important rule in our method of protein immobilization is that the constructs protein binding component must have one cysteine residue. The reasoning behind this is that our coil must form a disulfide bridge with a cysteine residue in an optimal orientation that allows ligand binding

and fluorescence to occur. If there is more than one cysteine, binding cannot be controlled and association with multiple coils may occur.



Cysteine immobilization modifications of binding proteins. Protein structures were obtained from the RCSB Protein Data Bank. All protein residues are shown in a blue cartoon preset with cysteine residues in red cartoon, respectively, using PyMOL. A. Phosphate binding protein with a Cys 256 modification (red). B. Potassium binding protein with a Cys 26 modification (red). C. Parathyroid hormone receptor, with a Cys 48 residue (red). D. Glucose/Galactose binding protein with a Cys 190 modification (red). E. Alpha-klotho (FGF23 receptor) with a Cys 664 modification (red).

One possible scenario is that your protein will have no cysteine residues existing already. This was the case with our phosphate binding protein. In this scenario we advise looking for an alpha helix as far from the active site and termini's as possible as to not interfere with binding or fluorescence. This selection is very hard to do without modelling or visualization software (14). Using the intensive BLAST results, select a conserved residue on that alpha helix that faces outwards towards solution and mutate this residue to a cysteine by altering the DNA codon for the residue (11,12). Ideally this cysteine will form a disulfide bridge with a coil when it is time to assemble your biosensor. Our group cannot directly comment on mutation of a beta-sheet to form a cysteine since

this has not been necessary (yet) for any of the binding proteins we have used. Ideally, we would assume the residue mutated would follow the same principles of facing outwards and being conserved. We have also avoided mutating intrinsically disordered residues to cysteines. In our molecular dynamic simulations, we often found these residues moved as hinges when binding ligand, and our fear was impeding on this hinge movement by addition of a bound coil.

A second scenario that may occur is you have a binding protein present with preexisting cysteine residues. Remember no disulfide bonds should exist as these proteins were eliminated in the screening process*. In this scenario your end goal is to have one final cysteine residue, outward facing, away from the active site and termini's, in a conserved region of the protein. To do this, use the BLAST search and visualization software to add this cysteine residue (11,12,14). Existing cysteine residues must be mutated using a BLAST search (11,12). In this method we replace the cysteine residue with another residue that appears consistently in the BLAST search at that corresponding position. Should there be no consistent residue appearing at this position we follow these rules:

1. If the cysteine is outward facing replace with serine (similar size and hydrophilic)
2. If the cysteine is in the interior of the protein replace with an alanine residue

One potential hazard is mutating a cysteine in the active site of a protein that directly interacts with a ligand. We have not seen this yet in any protein we have screened or used, however, should this exist it would be wise to perhaps eliminate this protein option in the screening phase.

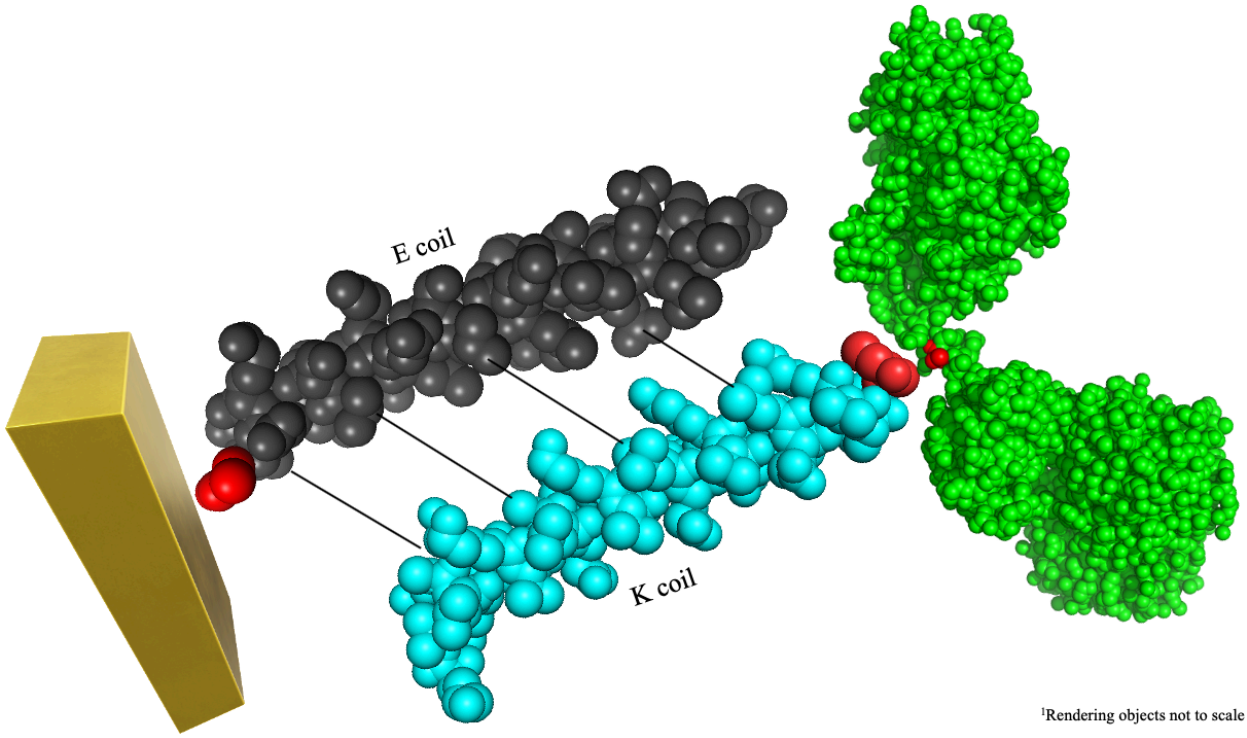
Assembly of the protein immobilization construct(s).

Assembly of the protein construct to the coiled coil system and biosensor surface should be performed stepwise as described in this protocol. These guidelines are important to adhere too as certain components must be isolated from each other to avoid the biggest concern, the formation of unwanted disulfide bridges.

1. Express the E coil, K coil, and fluorescent binding protein construct all separately. Our design for this was inserting the plasmid into E.coli, letting it replicate for an extended period of time, and then isolating that protein.
2. Addition of one coil to the gold biosensor surface. In the case of our biosensor design we use a gold-plated surface for the inside. It is not as expensive as it sounds. One coil, either E or K, should be added and that coils terminal cysteine will associate with the gold surface. This covalent bond is strong, and this coil will eventually anchor the other coil. A buffer should be used to remove free non-associated coils or coils that randomly associated with one another.
3. In a separate environment mix the other coil with the fluorescent protein construct. Ideally the two cysteines should associate and form a disulfide bridge. It is important as well in this step to remove any non-associated coils or proteins with a buffer. Size exclusion chromatography may be used to eliminate coils that associated with one another (18).
4. Flow the fluorescent binding protein solution into the biosensor. The fluorescent binding protein bound to the coil should not have any free cysteines that can bind to the gold surface

of the biosensor. It is expected that the E and K coils will associate with one another, thus immobilization is present (4).

Fluorescence can be detected using a UV-VIS spectrophotometer. Free ligand of known concentration can be used to generate a standard concentration curve.



E/K coiled coil, cysteine protein immobilization system. E and K coil five heptad repeats are displayed in dark grey and cyan, respectively. Red residues represent cysteine residues forming covalent linkage between coil & gold and coil & protein. Protein images were generated using PyMOL[1].

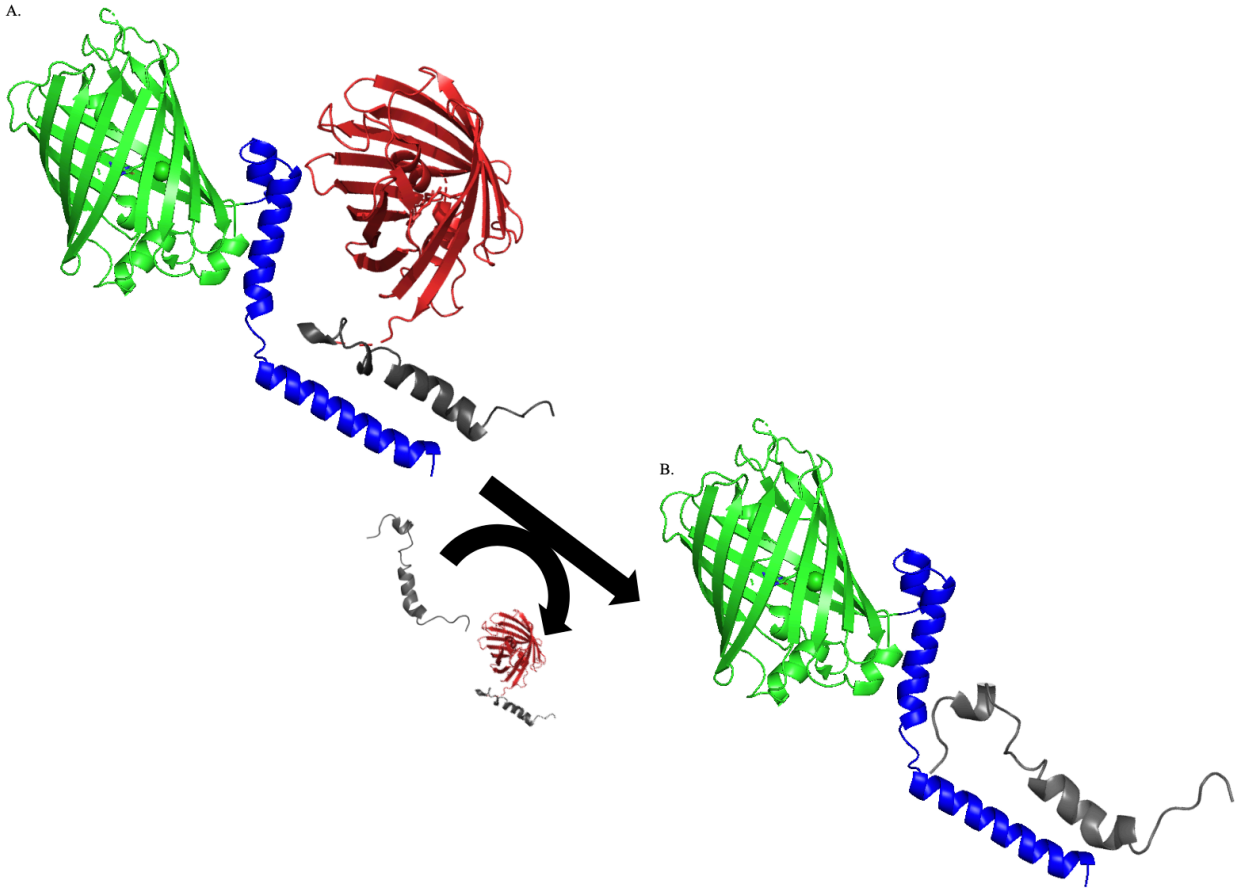
Parathyroid Hormone Receptor: A unique E/K coiled coil construct

Our group, after consultation with researchers and Nephrologists choose to design a protein construct that would assess the concentrations of parathyroid hormone (PTH) in patients with CKD. PTH is a prominent biomarker used to assess renal function and the course of CKD (e.g. early vs. end-stage) (19,20). PTH binds the parathyroid hormone receptor (PTHR), and in a typical human cell, would elicit a nuclear response (21).

Ultimately our research found that PTHR is a membrane receptor, which was a red flag when it comes to protein expression, as membrane proteins are notoriously more difficult to express (22). However, a recent study did assess the use of PTHR and FRET to measure the concentrations of PTH (21). This method did not go as far as to include the detection of PTH in a biosensor. Using the 60 amino acid N- terminal, extracellular domain, of PTHR to bind PTH, this would allow for easier protein expression (21). We added a mNeonGreen fluorophore to the N-termini of this extracellular domain. This domain had one cysteine and so adding our E/K coiled coil system that immobilizes the protein was not a large concern.

The issue at hand is rather detecting the concentration of PTH in the body. While PTH is difficult to express, a PTH fragment (residues 1-34) is easier to express (or obtain). This PTH fragment is attached to a mCherry fluorophore through its one lysine residues amino group (21). Allowing the PTH fragment with mCherry and PTHR with mNeonGreen to associate produces a FRET signal. When PTH from the body enters the biosensor, it displaces the PTH with mCherry and a drop in FRET signal is observed. You may consider this as a competition assay or similar to an antibody detection system.

This tool may be very powerful for the detection of other peptide hormones or receptor-ligand interactions where no conformational change is readily observed. A potential limitation however is that many peptide hormones in the human body exist in small concentrations. Since PTH is readily quantifiable at high concentrations in CKD patients this was not a problem, however, if the peptide hormone is known to exist at the pg/mL range, FRET may have difficulty detecting – and quantifying – the amount of peptide hormone. Our groups general recommendation if this is the case, would be to use an antibody or other method of detection.



Parathyroid hormone receptor competitive fluorescent assay. Protein structures were obtained from the RCSB Protein Data Bank. All protein residues are shown in a cartoon preset with the mNeonGreen, mCherry, parathyroid hormone receptor, and parathyroid hormone (1-34) as green, red, blue, and grey, respectively, using Chimera software. A. Parathyroid receptor hormone with mNeonGreen and parathyroid hormone with mCherry interaction. Fluorescent is expected to occur. B. Parathyroid hormone from the interstitial fluid enters the biosensor and competes with the

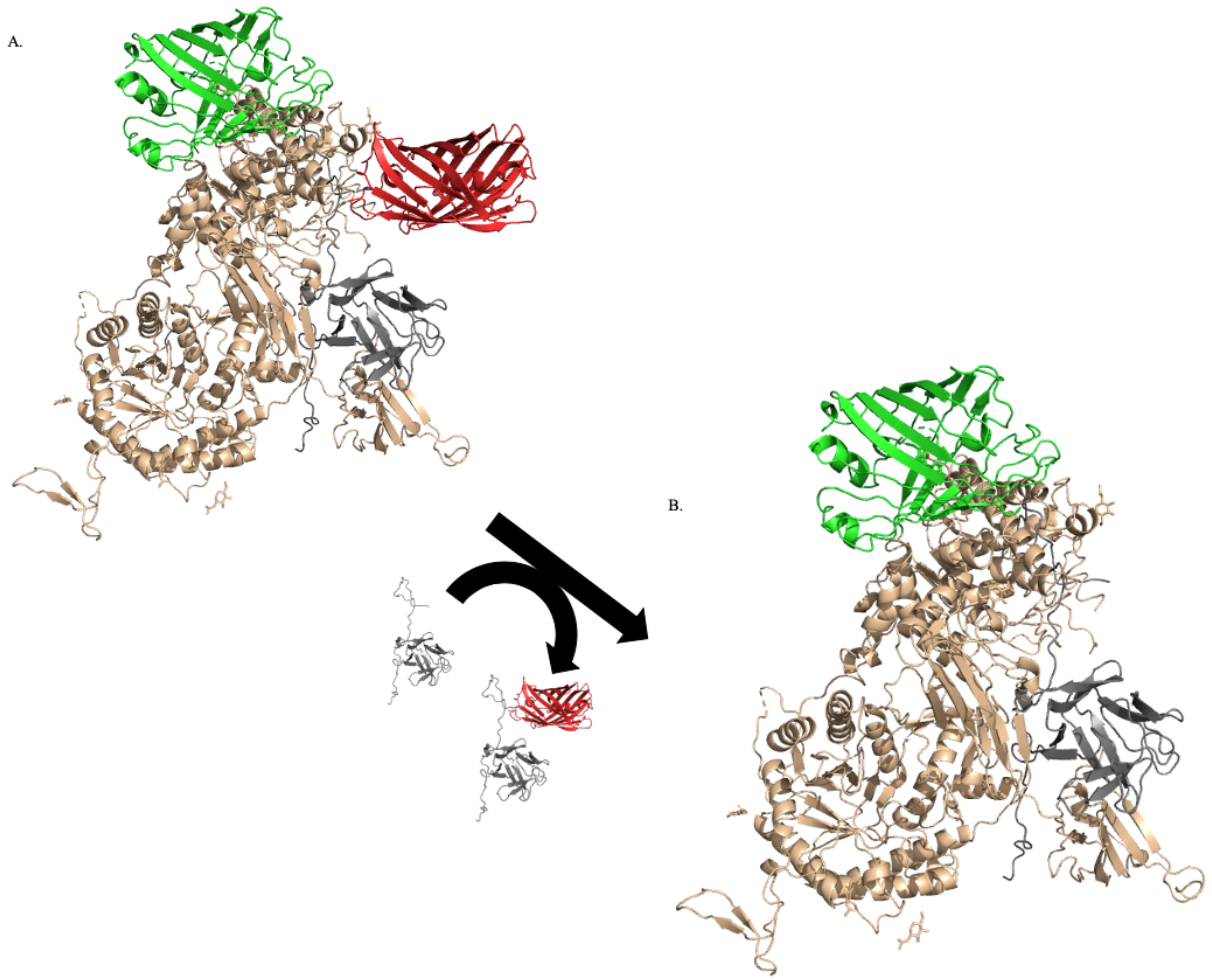
parathyroid hormone bound to mCherry. Interaction with parathyroid hormone receptor with mNeonGreen. Quantifiable drop in fluorescent intensity expected.

α-Klotho and FGF23: Managing lots of cysteine residues

Apart from PTH, FGF23 was also recommended in consultation with our physician and research partners as a biomarker to assess renal function and cardiovascular health. FGF23 binds to α-Klotho, a receptor protein, and suppresses phosphate reabsorption and vitamin D synthesis (23,24). Patients with CKD have remarkably higher levels of FGF23 compared to the general population, making it a valuable biomarker to monitor their care and disease progression (25).

α-Klotho has 12 cysteine residues, 4 involved in the formation of disulfide bridges and 1 involved in the coordination of a zinc ion cofactor (23). To attach our coiled-coil cysteine tail it was essential to remove the 7 remaining cysteines. Leaving the 5 cysteines involved in disulfide bonding and zinc ion coordination to associate during protein folding before the coiled-coil is added would ensure that the cysteine on the coiled-coil does not bind these residues. The cysteine that coordinates the zinc ion has an accessible surface area of about 4 angstroms, eliminating our concern of the coiled-coil accidently associating with it.

Alike the rest of our proteins, a L664C mutation on α-Klotho ensured we were placing the coiled-coil away from active site and in a position not interfering with fluorophore association (23). Because there is no evidence of α-Klotho undergoing a conformational change upon binding of FGF23, our group employed the same competition assay FRET type detection system as demonstrated previously in this paper with PTH and PTHR (21). In this case, mNeonGreen fluorophore is attached to α-Klotho and associates with FGF23 (peptide hormone) attached to an mCherry fluorophore. As FGF23 enters from the interstitial fluid, FGF23 attached to mCherry dissociates and the drop-in intensity correlates to the concentration of FGF23 from the interstitial fluid.



Alpha-Klotho (FGF23 receptor) competitive fluorescent assay. Protein structures were obtained from the RCSB Protein Data Bank. All protein residues are shown in a cartoon preset with the mNeonGreen, mCherry, alpha-klotho ternary complex, and FGF23 as green, red, wheat, and grey, respectively, using Chimera software. A. Alpha-Klotho with mNeonGreen and FGF23with mCherry interaction. Fluorescent is expected to occur. B. FGF23 from the interstitial fluid enters the biosensor and competes with the FGF23bound to mCherry. Interaction with alpha-klotho with mNeonGreen. Quantifiable drop in fluorescent intensity expected.

References:

1. Bajar BT, Wang ES, Zhang S, Lin MZ, Chu J. A Guide to Fluorescent Protein FRET Pairs. Sensors [Internet]. 2016 Sep 14 [cited 2020 Jun 2];16(9). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5038762/

2. Webster AC, Nagler EV, Morton RL, Masson P. Chronic Kidney Disease. Lancet Lond Engl. 2017 25;389(10075):1238–52.

3. Chen TK, Knicely DH, Grams ME. Chronic Kidney Disease Diagnosis and Management: A Review. JAMA. 2019 01;322(13):1294–304.

4. Chao H, Bautista DL, Litowski J, Irvin RT, Hodges RS. Use of a heterodimeric coiled-coil system for biosensor application and affinity purification. J Chromatogr B Biomed Sci App. 1998 Sep 11;715(1):307–29.

5. Mukherjee P, Banerjee S, Wheeler A, Ratliff LA, Irigoyen S, Garcia LR, et al. Live Imaging of Inorganic Phosphate in Plants with Cellular and Subcellular Resolution. Plant Physiol. 2015 Mar 1;167(3):628–38.

6. Shen Y, Wu S-Y, Rancic V, Aggarwal A, Qian Y, Miyashita S-I, et al. Genetically encoded fluorescent indicators for imaging intracellular potassium ion concentration. Commun Biol. 2019;2:18.

7. Mank M, Reiff DF, Heim N, Friedrich MW, Borst A, Griesbeck O. A FRET-based calcium biosensor with fast signal kinetics and high fluorescence change. Biophys J. 2006 Mar 1;90(5):1790–6.

8. Thomas KJ, Sherman DB, Amiss TJ, Andaluz SA, Pitner JB. A long-wavelength fluorescent glucose biosensor based on bioconjugates of galactose/glucose binding protein and Nile Red derivatives. Diabetes Technol Ther. 2006 Jun;8(3):261–8.

9. Choi M, Yamada S, Makishima M. Dynamic and ligand-selective interactions of vitamin D receptor with retinoid X receptor and cofactors in living cells. Mol Pharmacol. 2011 Dec;80(6):1147–55.

10. Burley SK, Berman HM, Kleywegt GJ, Markley JL, Nakamura H, Velankar S. Protein Data Bank (PDB): The Single Global Macromolecular Structure Archive. Methods Mol Biol Clifton NJ. 2017;1607:627–41.

11. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990 Oct 5;215(3):403–10.

12. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc. 2015 Jun;10(6):845–58.

13. Chen X, Zaro JL, Shen W-C. Fusion protein linkers: property, design and functionality. Adv Drug Deliv Rev. 2013 Oct;65(10):1357–69.

14. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem. 2004 Oct;25(13):1605–12.

15. Røkke G, Korvald E, Pahr J, Oyås O, Lale R. BioBrick assembly standards and techniques and associated software tools. Methods Mol Biol Clifton NJ. 2014;1116:1–24.

16. Alnahhas RN, Slater B, Huang Y, Mortensen C, Monk JW, Okasheh Y, et al. The case for decoupling assembly and submission standards to maintain a more flexible registry of biological parts. J Biol Eng. 2014;8(1):28.

17. Skrlj N, Erculj N, Dolinar M. A versatile bacterial expression vector based on the synthetic biology plasmid pSB1. Protein Expr Purif. 2009 Apr;64(2):198–204.

18. Burgess RR. A brief practical review of size exclusion chromatography: Rules of thumb, limitations, and troubleshooting. Protein Expr Purif. 2018;150:81–5.

19. Kritmetapak K, Pongchaiyakul C. Parathyroid Hormone Measurement in Chronic Kidney Disease: From Basics to Clinical Implications. Int J Nephrol. 2019;2019:5496710.

20. Souberbielle J-CP, Roth H, Fouque DP. Parathyroid hormone measurement in CKD. Kidney Int. 2010 Jan;77(2):93–100.

21. Nie Y, Li J, Liu Y, Zhang Q, Ma Q. A Visual FRET Immunofluorescent Biosensor for Ratiometric Parathyroid Hormone (1-84) Antigen Point-of-Care Detection. J Fluoresc. 2020 Mar;30(2):329–34.

22. Ishchenko A, Abola EE, Cherezov V. Crystallization of Membrane Proteins: An Overview. Methods Mol Biol Clifton NJ. 2017;1607:117–41.

23. Chen G, Liu Y, Goetz R, Fu L, Jayaraman S, Hu M-C, et al. α-Klotho is a non-enzymatic molecular scaffold for FGF23 hormone signalling. Nature. 2018 25;553(7689):461–6.

24. Erben RG, Andrukhova O. FGF23-Klotho signaling axis in the kidney. Bone. 2017 Jul;100:62–8.

25. Rodelo-Haad C, Santamaria R, Muñoz-Castañeda JR, Pendón-Ruiz de Mier MV, Martin-Malo A, Rodriguez M. FGF23, Biomarker or Target? Toxins. 2019 22;11(3).