1 **Modular Construction of Multi-Subunit Protein Complexes using Engineered Tags and**
2 **Microbial Transglutaminase**

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Abstract:

Motivations for the hierarchical assembly of protein complexes are diverse spanning biosensing, biomedical and bioreactor applications. The assembly processes should be simple, scalable, versatile, and biologically benign to minimize loss of component parts. A “plug and play” methodology comprising a generic linking apparatus may enable rapid design and optimization. One application that desires these qualities is metabolon construction wherein multiple enzymes are organized in defined pathways to mediate biochemical flux. Here, we propose a modular design by incorporation of crosslinking-compliant amino acid tags comprised of lysine or glutamine residues at the N- or C-termini of the to-be-assembled proteins. These amino acid tags enable covalent crosslinking using microbial transglutaminase (mTG). Modularity is demonstrated where stoichiometries and relative positions of enzymes and other functional proteins are altered. Construction of multifunctional complexes are demonstrated by crosslinking domains of different function and origin. Namely, we built a two-subunit quorum sensing (QS) biosynthetic metabolon on solid supports and altered stoichiometries of the limiting constituents to increase the overall rate of reaction. To display functionality beyond biosynthesis, we constructed a molecular communication ‘device’ (antibody binding Protein G–QS complex) to target bacterial cells and demonstrated tailored QS responses among targeted bacteria. We propose that this approach, solid phase mTG-mediated linkage of biological components, can be used for assembly within many environments including microreactors or lab-on-a-chip systems. Because the methodology is general, we envision construction of multi-functional protein complexes in a ‘plug and play’ fashion for a variety of biosensing and synthetic biology applications.

1. Introduction

Biofabrication is the assembly of biological components such as cells, tissues, proteins, polysaccharides, lipids, nucleic acids etc., using biological means or mimics thereof for the creation of devices and other functional constructs. These biological structures are often integrated within microfluidic and microelectronic systems where they execute an array of functions, including the biosensing of environmental samples [1-3] and toxicity and efficacy testing in lab-on-a-chip devices [4-7]. Advances in genetic and protein engineering have enabled the design and construction of pathways that catalyze a diverse range of reaction products in an efficient manner [8, 9]. Most of these multistep biotransformation processes are located inside the cells where enabling cofactors and effector molecules are also nearby. These networks, called metabolons, mediate a variety of enzymatic reactions and their construction and assembly can involve extensive metabolic engineering of the host. *In vitro* methods often feature simplified product recovery and minimal substrate toxicity [10, 11], but can suffer by providing limited access to needed co-factors and other effectors. Methods used for the *in vitro* construction of metabolons are many, including co-localization of enzymes via covalent crosslinking and entrapment of enzymes into nano structures or containers [12, 13]. Additionally, immobilization on solid supports, such as protein scaffolds where enzyme modules are engineered for selective affinity to the scaffolds have appeared [14, 15]. While advantages of crosslinking over entrapment include active site proximity and reduced resistance for substrate or product transport, there are few means to enable domain-specific assembly of component parts. Genetic methodologies can provide control over subunit arrangement and conformation[16], some require long linker peptides as well as extensive selective binding domains.

1 In this work, we enable metabolon construction by engineering subunits with simple, short
2 linker tags (5-7aa). This linking system exploits the specificity of the crosslinking enzymes for
3 assembly so that the tags provide for orientation of the to-be-assembled subunits. We
4 demonstrate this concept by the assembly of two bacterial quorum sensing (QS) synthases, S-
5 Ribosylhomocysteinase (LuxS) and S-Adenosyl homocysteine nucleosidase (Pfs). We further
6 connected these enzymes to protein G, a *Streptococcal* bacterial protein that binds to the Fc
7 region of IgG [17]. The QS enzymes act to convert S-Adenosyl homocysteine (SAH) to
8 homocysteine (HCY) and autoinducer-2 (AI-2), a QS signal molecule. We then targeted the
9 protein G-QS enzyme complex to bacterial cells using anti-*E.coli* antibodies leading to
10 biochemical reactions on surface of bacteria and site-specific delivery of the autoinducer to the
11 cells.

12 In Fig. 1, we depict the overall scheme for protein assembly. In Fig. 1A, the first subunit is
13 selectively bound to a solid support (Co^{2+} resin) via an N-terminal hexahistidine tag. Then, the
14 second subunit engineered with lysine tags on both termini is covalently grafted using mTG onto
15 the glutamine tag of the first subunit (Q tag). The reaction mechanism is shown in Fig. 1B. Then,
16 in Fig. 1A, we depict linkage of a third subunit comprised of a Q-tag also on the C-terminus for
17 the eventual assembly of many structures covering the bead. In all cases, microbial
18 transglutaminase (mTG) is used to graft adjacent glutamine and lysine residues forming a trans-
19 peptide bond [18-25]. The linker tags engineered onto subunits Pfs, LuxS and Protein G were
20 either hepta-lysine (7aa) and/or penta-glutamine (5aa) [21] and crosslinking was mediated by the
21 addition of microbial transglutaminase (mTG). Schemes for each protein, its tags, and cleavage
22 of the histidine tags are included in Fig. 1C. After using these methodologies to make complexes,
23 we evaluated enzymatic flux for the production of AI-2. We note that LuxS can be rate limiting

[26, 27]; we altered stoichiometry to generate a Pfs-LuxS-LuxS complex, our hypothesis being that the overall rate might be increased. Finally, as noted above, we added a protein assembly domain, Protein G thereby creating Pfs-LuxS-Protein G complex that could be released from the Co²⁺ resin and further complexed with an antibody to recognize bacteria. We targeted the released complexes to bacterial cells and elicited quorum-sensing responses from bacterial populations; our hypothesis being a change in phenotype that was correlated to the AI-2 synthesis flux through the complex.

2. Materials and Methods

2.1. Plasmids and Strains:

All plasmids and strains used in this study are listed in Table S1. We performed all cloning and transformations as per standard protocols [28]. Clone designation includes the tag and the employed terminus (e.g., pTrcHisA-7K-LuxS-7K indicates a LuxS vector with a 7-residue lysine tag at both the N- and C- termini, the expressed protein is denoted κL_K). All primers (Integrated DNA Technologies) used for engineering of proteins in this study are summarized in Table S2. All the engineered DNA sequences utilized Xho-1 and EcoR1 restriction sites at 5' and 3' ends of the DNA fragments. For cloning purposes, DNA was ligated with TOPO Blunt II vector (Life Technologies) and transformed into TOP 10 cells. Plasmids were sequenced at the DNA Core Facility of the Institute for Bioscience and Biotechnology Research. For expression, genes were inserted into pTrcHisA plasmid (Lab stocks) and transformed into respective expression hosts. We transformed LuxS containing clones into RK 4353 (*pfs*⁻) for expression of LuxS proteins and the BL21 (*luxS*⁻) for expression of Pfs proteins. A His₆ tag is part of the pTrc plasmid backbone; all expressed proteins have N-terminal His tags. Importantly, all His tagged proteins also contain an enterokinase cleavage site located between the His tag and the K/Q linker tag in the primary sequence (Fig. 1C).

1

2 We grew the expression hosts in LB media (Sigma-Aldrich) and induced at OD₆₀₀ of 0.4 with 1
3 mM IPTG (Sigma). After 4 hrs at 30°C, cells were centrifuged and resuspended in 15 mL of 10
4 mM phosphate buffered saline (PBS), pH 7.4 (Sigma) and sonicated for 15 min. Post sonication,
5 lysed cells were centrifuged, soluble proteins were retained and purified via HiTrap columns (GE
6 Healthcare). Purified proteins were subsequently dialyzed with PBS and stored at -20° C. We
7 selectively removed His tags by incubation with enterokinase overnight at 4° C as per
8 manufacturer's specifications (EK-Away, Invitrogen). To ensure robust transglutaminase
9 reactions, we rewashed all proteins using a second round of Co²⁺ resin. This served as a quality
10 control to ensure His tag removal. Proteins were stored for further use at -20° C.

11 **2.3. Conjugation of Engineered proteins using mTG:**

12 We aliquotted 20µl of Talon Metal Affinity resin (Clontech) in a centrifuge tube and
13 centrifuged to remove residual buffer. To the pelleted resin, we added 10 µl His-tagged protein
14 (concentration varied as noted) with C-terminal 'Q' tags. These were mixed well to mediate
15 attachment of 'His' tagged proteins to resin. Next, we added 10 µl of mTG (Ajinomoto, enzyme
16 activity of 12 U/mg) at concentrations to reflect the desired molecular ratio between reacting
17 protein and mTG (indicated as β), and mixed well at RT. Immediately, we also added 10µl of
18 reacting 'K' tagged protein (concentration varied with experiments) to the protein-resin mix and
19 incubated with shaking at room temperature for 60 min. After incubation, we washed the resin
20 3x with PBS to remove unbound proteins and residual mTG. In cases where a third subunit was
21 attached, we added another round of subject protein with reaction mixtures exactly as before. To

elute the conjugated proteins, we treated the resin with PBS+200mM Imidazole for 10 min on ice and later centrifuged to remove the eluted protein complexes from beads.

2.4. Measurement of Homocysteine using Ellman's Assay and Cyclic Voltammetry:

We measured homocysteine using calorimetric Ellman's assay as described in [17] and electrochemically using cyclic voltammetry (CV) [30].

2.5. Construction of Antibody-Protein complexes:

We built the Pfs-LuxS-Protein G complexes on beads as described above. To characterize quantities, we added 10 µg of FITC-labelled anti-*E.coli* antibodies (AbD Serotec) to the protein G-enzyme complexes on the beads and incubated at RT for 30 min. We washed three times using PBS to remove non-specifically or unbound antibodies. Later we eluted the antibody-bound Protein G-enzyme complexes from beads as described before and measured their fluorescence using a plate reader (SpectraMax). The final reaction volume was 200 µl for fluorescence measurements.

2.6. Flow Cytometry measurements for targeted enzyme reactions:

To ascertain deployment capabilities of our constructed complexes, we eluted the Protein G-Pfs-LuxS complexes from beads, incubated them with FITC labelled anti-*E.coli* antibodies for 30 minutes, and quantified them via fluorescence microscopy and FACS. We grew *E. coli* CT104 cells [29] with pCT06 and pET-DsRed plasmids (Table S1) overnight at 30°C (they can respond to AI-2 by inducing DsRed expression [29]). We pelleted 0.1 OD of cells, incubated with 5% BSA for 30 min, and washed three times with PBS. We next incubated the cells with Antibody-Protein G-Enzyme complexes for 30 min and washed 3x with PBS to remove unbound

antibodies and enzyme complexes. We pelleted the cells and incubated them with DPBS (Dulbecco's Phosphate buffered saline, Sigma) solution at 37°C without shaking. After 4 hours, we centrifuged the cells and analyzed using flow cytometry. We used a minimum of 20,000 cells for analysis of each sample. We used FlowJo software for analysis and image generation[17].

3. Results

3.1. Reactivity of engineered K and Q tags mediated by mTG

We first studied the effect of engineered tags on the crosslinking of proteins freely suspended in buffer. In Fig. 2A, we incubated mTG with enzymes LuxS (L) and Pfs (P) without linker tags (Panels 1 and 2), LuxS with C-terminal K and Q tags (Panels 3 and 4), and Pfs with C-terminal K and Q tags. We used a β of 100 (β , the molar ratio of reacting subunit to mTG) and incubated at RT for 0, 15, 30 and 60 minutes (i.e., four lanes are depicted). We stopped the reactions via heat denaturation at 95°C for 5 minutes and assayed crosslinking by SDS-PAGE and Western blot using anti-His antibodies. We note that untagged LuxS and Pfs (L and P, respectively) exist as homodimers in their native states [31, 32] and without mTG treatment appear as monomers on gels under denaturing conditions. With mTG, native L and P formed small quantities of dimers after ~30-60 minutes (panels 1 and 2). Next, we incubated ‘K’ and ‘Q’ tagged LuxS (L_K and L_Q) with mTG under identical conditions as the native enzymes (panels 3 and 4). As noted in Methods, the prefix and suffix indicate tags at N and C termini, respectively. For example, L_K indicates LuxS with ‘K’ tag at the C terminus. While L_K formed dimers more readily than native L, L_Q actively cross-linked to form oligomers. The arrows indicate dimer, trimer and tetramer bands; the quantity of each increased with incubation time. Interestingly, Pfs with ‘K’ and ‘Q’ tags (P_K and P_Q , panels 5 & 6) reacted analogously to L_K and L_Q , but with less oligomerization for the ‘K’ tag. While only showing two proteins, these data suggest the ‘Q’ tag is advantageous for mTG-mediated protein oligomerization. That is, ‘Q’ tagged protein cross-linked to itself more than untagged native proteins and those with ‘K’ tags.

We next studied how β (molar ratio of the subunits to mTG) played a role in determining the extent of crosslinking of the engineered proteins. Fig. 2B depicts mTG-mediated crosslinking of

P_Q using mTG with varied β at two time points, 15 and 60 minutes. With $\beta = 1$ (same amount of protein as mTG), a smear of P_Q was formed in both samples, whereas with $\beta = 100$ we observed well-defined dimers and trimers which increased with time. With $\beta = 1000$, P_Q was relatively unreactive at 15 minutes and formed dimers after 60 min. Similar time and molar ratio profiles of other engineered proteins are provided in Supplementary Fig. S1. In sum, these results indicate that β , the ratio of Q-tagged protein to transglutaminase influences the extent of oligomerization. The mechanisms by which this appears to function are unclear, but the trends observed could be anticipated (e.g., more enzyme, more crosslinking).

We next tested the kinetics associated with conjugating two different enzymes together and whether the linked AI-2 synthesis pathway could function to make AI-2. In this case, we employed both ‘Q’ and ‘K’ tagged proteins. We also note that Pfs-LuxS chimera have been expressed as an intact fusion protein in *E. coli* and when purified and resuspended in buffer with SAH, produces AI-2 [17]. Thus, the test here evaluates whether LuxS and Pfs can be crosslinked using the engineered ‘K’ and ‘Q’ to produce AI-2. Both P_Q and L_K were reacted with mTG with a β of 100 for 60 minutes (Fig. 2C). We found a preponderance of homodimers, rather than heterodimers when cross-linked in solution.

To explore whether we could promote heterodimer formation and selectively remove homodimers, we first assembled one ‘anchor’ protein on Co²⁺ affinity resin using the N-terminal His tag, the aim being to restrict access to the N-terminal fusion tag. We then cross-linked the second protein to the first while on the bead using the mTG and associated fusion tags. For this, the second subunit was treated with enterokinase (EK) to remove N-terminal His tags (Methods), precluding subsequent His-mediated assembly on the Co²⁺ resin. After reaction with mTG for 60 minutes, we washed the beads 3x with PBS to remove unreacted subunits and mTG. We then

1 eluted the cross-linked subunits from beads via ion exchange (Methods) and assayed using
2 Western blots. In lane L1 of [left blot in Fig. 2D](#), unreacted P_Q was loaded as control. In lane L2,
3 we reacted P_Q with mTG for 60 min with a β of 100 and observed oligomerization as in the case
4 of reactions in solution (Fig. 2C). In L3, we show heterodimer formation on the beads by the
5 creation of P_Q-L_K. That is, to the bead bound first subunit, P_Q, a second subunit engineered with
6 'K' tags was added (in this case _KL_K; LuxS with 'K' tags at both N and C termini) along with
7 mTG at β of 100. P_Q-_KL_K heterodimers were revealed as a lighter band than the homodimers
8 (e.g., P_Q2). In addition to P_Q-_KL_K, we observed P_Q2-_KL_K heterotrimers, all indicating crosslinking
9 of Pfs and LuxS enzymes. Control reactions where the second subunits had no engineered tags
10 yielded no heterodimers indicating the importance of engineered lysine tags to mediate
11 heterodimer formation (Supplementary Fig. S2). As expected, the lack of mTG for crosslinking
12 also yielded no heterodimers (Supplementary Fig. S2). Under these crosslinking conditions with
13 β = 100, by image analysis we estimate that ~25% of Pfs enzymes were crosslinked to LuxS
14 (Supplementary Fig. S3). [The crosslinking percentage can be improved by employing \$\beta\$ of 1.](#)
15 [However, in this condition the ability to discern heterodimers and homodimers under denaturing](#)
16 [SDS gel is diminished \(as seen in Fig. 2B and S1\). To decrease side product formation, we](#)
17 [switched the positions of Lys and Gln tagged proteins in our bead-based system \(Fig. 2D, blot on](#)
18 [the right\). We observed a reduction in nonspecific product formation in comparison to when Gln](#)
19 [tagged proteins were immobilized on the bead.](#)

20 In summary, these results indicated that lysine and glutamine rich tags engineered onto the
21 C- and N-termini of the two AI-2 synthases enabled crosslinking, but additional measures
22 (binding to Co²⁺ spheres) were needed to guide linkages to form heterodimers. That is, selective

immobilization on beads when combined with engineered tags and mTG, lead to somewhat improved formation of heterodimers, but exclusivity was not demonstrated.

mTG mediated control of metabolic flux

We next explored the capability of these assembled enzymes to mediate metabolic flux. Pfs and LuxS are bacterial enzymes involved in the activated methyl cycle and act together to convert S-Adenosyl homocysteine (SAH) into homocysteine (HCY) and (S)-4,5-dihydroxy-2,3-pentanedione (DPD) in a two-step process [33] ultimately yielding autoinducer-2 (AI-2) which is normally secreted by bacteria (Fig. 3A). We hypothesized that (i) the engineering tags would not destroy activity, and (ii) that proteins coupled by mTG would retain activity providing complete pathway synthesis. Further, we hypothesized that the proximity of the coupled enzymes could provide efficient substrate utilization.

In Fig. 3B, we immobilized 3 μ M ‘Q’ tagged Pfs on Co²⁺ beads as before and crosslinked with 3 μ M of corresponding ‘K’ tagged second subunit (L_K) by mTG (as in Fig. 2D). In this case, to ensure crosslinking, we incubated reactants at RT for 60 min and $\beta = 1$. As before, we washed the beads 3x with PBS to remove unreacted enzyme and mTG. We then incubated bead bound enzyme complexes with 1mM SAH at 37°C for 60 min. We measured homocysteine (HCY) using a colorimetric assay (Ellman’s sulfhydryl assay, see Methods). Homocysteine levels were markedly different in samples with and without mTG. When coupled with Western oligomerization data, these results indicate that mTG-crosslinked LuxS and Pfs function to make homocysteine.

We next studied if we could change the yield of homocysteine by varying the subunit quantities in the crosslinked complexes (Fig. 3C). With bead-bound P_Q held constant, we varied

1 the concentrations of L_K and crosslinked exactly as before. After washing 3x with PBS, the
2 beads were incubated with 2mM SAH for 60 minutes and HCY generated was measured.
3 Importantly, we found a linear increase in HCY generated with incubated LuxS concentration,
4 suggesting LuxS rate limitation. As controls, we performed exactly analogous experiments using
5 native L (without 'K' tag), and there was no significant accumulation of HCY indicating
6 minimal non-specific binding of the LuxS to either the resin or the loaded Pfs. This also
7 indicated that mTG preferentially recognized 'K' and 'Q' tags more than any exposed lysine or
8 glutamine residues on the enzyme subunits. Having demonstrated L_K reacted with Co^{2+}
9 immobilized P_Q , we next studied how bi-tagged LuxS (${}_KL_K$, LuxS with 'K' tag at both N and C
10 termini) could crosslink to P_Q . Again, as before, we crosslinked ${}_KL_K$ with P_Q using mTG. We
11 then provided SAH and observed for HCY generated (See Fig. 2 in ref [34]). Interestingly, the
12 HCY doubled, perhaps suggesting an advantage to having 'K' residues on each end of the
13 enzyme. Western blots (See Fig.1 in ref [34]) indeed showed enhanced crosslinking of ${}_KL_K$ to P_Q
14 (relative to L_K); correlating with HCY yields determined by Ellman's assay.

15 In Fig. 3D, we sought to assemble Pfs-LuxS-LuxS trimers on beads to enhance LuxS activity
16 relative to Pfs, thereby increasing the overall reaction rate. To the P_Q - ${}_KL_K$ complex initially built
17 on beads, we added L_Q and mTG for a second round of crosslinking exactly as before, expecting
18 that any unreacted lysine tags on ${}_KL_K$ could crosslink to L_Q . After washing 3x, we provided 1mM
19 SAH, observed for HCY yields using Ellman's assay after incubation for 60 min at 37°C. As
20 anticipated, putative three-subunit complexes enhanced the biochemical flux resulting in
21 increased generation of HCY. Negative controls with non-tagged L exhibited no increase in
22 homocysteine and importantly, an incremental increase in yield was observed as the second
23 incubation with LuxS was performed with higher LuxS concentration (from 1.5 to 3 μ M).

Subsequent increases in LuxS resulted in no net gain in reaction, suggesting matching of LuxS with Pfs activity. Also, we note that all three subunit complexes were built on the two-subunit complexes from the 3 μ M LuxS incubation, and in all cases the yields of all three subunit complexes were higher than the corresponding two subunit complexes on which they were built. As controls, we built analogous two and three subunit structures without mTG and all of them generated negligible yields of HCY indicating the crosslinking was mediated by mTG. Fig. 4 in ref [34] depicts HCY generation for an extended duration (240 min) from different Pfs-LuxS complexes. In these data, the three-subunit complex (P_{Q-K}L_K-L_Q) enabled faster reaction flux than the two-subunit complexes (P_{Q-K}L_K) which, in turn, were faster than the first P_Q-L_K complex. Controls for these experiments were again structures built without mTG and as expected, controls resulted in negligible reaction.

Interestingly, results from western blots were inconclusive for trimer construction, suggesting non-conformationally assembled enzymes. Thus, in Fig. 3E, we examined in more detail, the differences in HCY generated by the addition of third subunit. That is, we built two and three subunit complexes as before but in one case, we replaced the κ L_K with an *inactive* subunit (**I** κ L_K, obtained from periodic freeze thawing). The inactive subunit provided no enzyme activity, but should have retained its crosslinking capability. We built three-subunit complexes with the inactive LuxS as the second subunit exactly as before and compared HCY yields against controls. Specifically, we had a two-subunit inactive complex and a three-subunit complex built on the two subunit inactive complex without mTG. We provided these complexes with SAH and observed HCY in real time using cyclic voltammetry and the results obtained are correlated to homocysteine concentrations [29]. Results indicated that the highest flux was obtained for the Pfs-LuxS-LuxS trimer, as expected. The next highest reaction rate was observed for the Pfs-

LuxS dimer. Fluxes generated from the 3-subunit complexes built with an inactive second subunit LuxS (w & w/out mTG) were lower than the flux generated by two-subunit complexes built with active LuxS. It is noteworthy that the Pfs-inactive LuxS-LuxS construct exhibited almost exactly one half the activity of the Pfs-LuxS-LuxS construct. Additional tests were performed with varied Pfs (see Fig. 3D in ref [34]) where Pfs-Pfs-LuxS complexes were built and the flux generated from the Pfs-Pfs-LuxS complexes were lower than the Pfs-LuxS complex. We note, however, that in these cases the maximum activity was obtained by the addition of 6 μ M LuxS and this was similar to the maximum activity observed for the Pfs-LuxS construct built with 3 μ M LuxS, again suggesting LuxS rate limitation. In sum, these results could be rationalized by at least two factors: (1) the extra spacing (either partially inactive LuxS (Fig.3E) or Pfs (Fig.3 in ref [34])) between active Pfs and LuxS enzymes contributes to a reduced reaction rate; and/or (2) there is less binding of third subunits to second subunits than of second subunits to first subunits. Results from Fig. 3D illustrate that the addition of the third subunit depends on its concentration, thus some level of optimization is needed to maximize linkage to the second subunit and even after optimization, results may not show as much linkage as a second subunit to its first.

Thus, data in Fig. 3 demonstrate that we built two and three subunit crosslinked complexes on beads using engineered 'Q' and 'K' tags and mTG. The complexes assembled were shown to display altered reaction kinetics based on the number of rate-limiting enzymes. In addition, we found that as the number of desired enzymatic subunits to be crosslinked was increased, the per enzyme reaction rate decreased, suggesting need for further examination and/or a limitation of this approach.

3.2. Assembly of multi-functional complexes using mTG

Beyond linkage of biosynthetic enzymes, we next studied whether mTG mediated cross-linking could be used for the construction of multi-functional protein complexes, again in a flexible manner. In addition, we wanted to explore further the limitations found by sequentially attaching two LuxS enzymes. We constructed a Pfs-LuxS complex (as before) but then coupled this with *Streptococcal* protein G [35]. Protein G has the ability to bind the Fc region of IgG. We suggest this enables the assembly of covalently tethered complexes with targeting antibodies, enabling the localized generation of reaction products. In our case, we first sought to create a Pfs-LuxS-Protein G complex that could be coupled with anti-*E. coli* antibodies in order to direct the enzymes to the surface of *E. coli* (Fig. 4A) for localized AI-2 synthesis.

We first studied whether protein G could be crosslinked to Pfs-LuxS complexes using the same linking motif ('L' and/or 'Q' tags). That is, we sought to create Pfs-LuxS-Protein G complexes comprised of P_Q, K_{LK} and G_Q (protein G engineered with 'Q' tag at C terminus). We loaded P_Q on the beads and crosslinked the second subunit (K_{LK}) using mTG for 60 min at RT with $\beta = 1$. After washing the beads 3x with PBS, we crosslinked G_Q as before. We used Dylight 633-labeled protein G for visualization. Beads were again washed 3x to remove unbound protein G. Analogous to the LuxS-capped complexes, controls for these tests were two subunit complexes, incubated with labeled G_Q but without mTG. We eluted the assembled complexes from beads and measured fluorescence (Fig. 4B). We found the fluorescence from complexes built with mTG was proportional to the concentration of G_Q added, again suggesting crosslinking

1 limitation with protein G (as with LuxS previously). Interestingly, however, a doubling of
2 protein G yielded a doubling of fluorescence, suggesting that incremental protein G was fully
3 linked to the two subunit complexes. Analogous controls built without mTG had negligible
4 fluorescence indicating lack of G_Q crosslinking. Importantly, these results support our hypothesis
5 that the third subunit, G_Q, was bound to two-subunit complexes on the beads.

6 To further evaluate assembly, we added 10 µg of FITC-labelled antibodies to these three-
7 subunit complexes (unlabeled in this case). After incubation for 30 minutes, we washed the
8 beads 3x with PBS to remove unbound IgG and eluted the protein complexes from beads via ion
9 exchange. We measured fluorescence of the complexes “loaded” with the labelled antibodies
10 (Fig. 4C) and found a linear increase with antibody until ~5 µM, when the fluorescence appeared
11 to saturate. Corresponding controls consisted of the same incubations but without mTG; there
12 was minimal fluorescence in all samples indicating the absence of protein G crosslinking and the
13 lack of antibody assembly. These data support our original hypothesis that IgG bind protein G
14 that, in turn, are the terminal addition to P_Q-K_LK complexes. Moreover, the additional antibody
15 was nearly stoichiometrically similar to the added protein G (e.g., antibody, provided in excess,
16 was bound in proportion to the added protein G until 8µM, when fluorescence became saturated).
17 Being a ‘third’ subunit, these data suggest that a progressive inefficiency of subunit addition may
18 not have been a primary factor for the decreased flux observed in Fig. 3E (albeit this addition is
19 not enzymatically mediated).

20 We then tested whether the assembled three-subunit (P_Q-K_LK-G_Q) complexes retained
21 enzymatic activity. Three-subunit complexes were assembled on beads using mTG exactly as
22 before. Control tests were performed on two-subunit complexes without protein G (P_Q-K_LK). We
23 provided the bead bound complexes with substrate 1mM SAH, incubated for 60 minutes at 37°C

1 and measured HCY. We found $P_{Q-K}L_KG_Q$ complexes resulted in 4-fold lower homocysteine
2 levels than $P_{Q-K}L_K$ complexes (without protein G), suggesting that addition of Protein G to KL_K
3 resulted in a significant decrease in enzymatic activity ($p < 0.05$) (Fig. 4D). We then replaced
4 protein G with EGFP (E_Q , EGFP with Q at C terminus) to test whether this effect was subunit
5 specific; the decrease in HCY was similar (Fig. 5 in ref [34]) suggesting crosslinking of an
6 additional subunit to LuxS in a Pfs-LuxS two-subunit complex resulted in reduction in enzyme
7 activity. Recall that our previous tests suggested that LuxS was limiting, so we suspected the
8 additional subunit might have interfered with the first LuxS activity (e.g., linked on both
9 termini). We then changed the relative positions of the subunits creating a $G_Q-KP_KL_Q$ complex
10 where LuxS was added at the end. Thus, we assembled G_Q first and created $G_Q-KP_K-L_Q$ using
11 mTG exactly as before. In this configuration, HCY yields from $G_Q-KP_KL_Q$ were lower but not
12 significantly different from $P_{Q-K}L_K$ ($p > 0.05$) (Fig. 4D) indicating that the subunit re-
13 arrangement had rescued the original enzymatic activity. The slight decrease could have been
14 attributed to decreased linkage of LuxS as a third subunit. Overall, these data highlight the
15 flexibility of our modular construction approach for building multi-subunit complexes through
16 enzyme mediated termini-specific linkage.

17 **3.2.1. Deployment of Multifunctional Complexes**

18 We next tested if we could ‘build’ and ‘transplant’ our protein complex to antibody-targeted
19 sites where they could carry out enzymatic reactions. We built $P_{Q-K}L_KG_Q$ and $G_Q-KP_KL_Q$
20 complexes on the beads using mTG, as before. We eluted these protein complexes from the
21 beads and incubated with FITC labelled anti-*E.coli* antibodies. After 30 minutes, we added the
22 enzyme-antibody complexes to *E. coli* CT104 reporter cells (these cells respond to AI-2 with
23 DsRed fluorescent protein expression [29]). We note that AI-2 is a byproduct generated with

HCY in equimolar amounts by the enzyme complexes. After an incubation time of 30 minutes, we washed the cells 3x with PBS to remove unbound protein-antibody complexes and suspended the cells in DPBS (Dulbecco's Phosphate Buffered Saline) solution containing 1mM SAH at 30°C for 4 hours without shaking. We used FACS to analyze the bacterial populations for their DsRed fluorescence. Controls for these experiments were the cells incubated with protein complexes but without the anti-*E.coli* antibodies. Representative fluorescence microscopy images of the experimental and control bacterial populations are provided in Fig. 6 in ref [34]. Notably, populations incubated with antibody coupled enzyme complexes displayed ~15-fold more fluorescence overall than controls indicating that antibody bound complexes attached to bacterial cells, they were active, and synthesized AI-2 was delivered to the cells wherein they responded by altered gene expression (Fig. 4E). Between the populations coupled with antibodies and enzyme complexes, there were significant differences in Mean Fluorescence Intensities (MFI). That is, populations with Ab-G_{Q-K}P_{K-L}Q complexes had 6-fold higher mean fluorescence intensity than the populations with P_{Q-K}L_K-G_Q-Ab (Fig. 4E). The differences in AI-2/HCY yields from complexes G_{Q-K}P_{K-L}Q and P_{Q-K}L_K-G_Q measured in Fig. 4D correlated well with bacterial population's MFI in Fig. 4E. These results indicate that differences in enzyme activity that stem from the order of assembly were replicated by changes in gene expression of the cells exposed to the same complexes.

4. Discussion

In this work, we demonstrate a facile method for the assembly of multi-subunit protein complexes using engineered N- and C-terminal 'Q' and 'K' tags that are coupled using mTG on solid supports. We built a two-enzyme metabolon involving bacterial QS enzymes Pfs and LuxS and displayed control of metabolic flux across these enzymes. Through this method, we were also able to build a multi-functional Pfs-LuxS-Protein G complex that was eluted from assembly beads and used for specific targeted enzyme applications. We displayed modularity and versatility in construction by switching of positions of the subunits in the multi-subunit complexes.

Further, we observed that by varying reaction stoichiometries and protocols, different product trajectories could be developed. To minimize side product formation and drive heterodimer formation, factors that can be optimized include β (the ratio between subunit and mTG), the position of K and Q tagged proteins in the bead based crosslinking process, and the duration of crosslinking. β had a positive correlation with the extent of crosslinking overall. While we employed β of 100 for crosslinking to get 25% heterodimerization in Fig. 2D, we also observed decreased yield of crosslinked protein. On the other hand, we could decrease the β from

100 to 1 and obtain higher levels of protein, but at the cost of increased construct heterogeneity (Fig. 2).

Positions of Q and K tagged proteins also provide a basis for control. While the ‘Q’ tags caused self-oligomerization when treated with mTG, we did not observe the same with lysine (‘K’) tags (Fig. 2A). We reason that this behavior of Q tags can be attributed to the two-step reaction catalyzed by mTG, where first, the glutamines in the ‘Q’ tag can readily interact with mTG to form an intermediate complex that can then attack the lysines nearby. Considering that the proteins tested here exist as dimers, there is increased propensity to target lysines of their own leading to homo-oligomerization than target other enzymes or proteins. For the proteins engineered with ‘K’ tags, the glutamines present on the proteins are largely inaccessible to mTG, thereby making them inert to intra-crosslinking [36]. Hence, when positions of Q and K tagged proteins are switched, with K tag on the bead and Q tag in solution, the K tag is inert to mTG leading to reduced side products. Once the soluble Q tagged second subunit is added, mTG can react with its glutamine tag and mediate crosslinking. However, the percentage of crosslinking can remain low because the mTG-Gln intermediate complex can potentially crosslink to any soluble lysine, get washed away, or get hydrolyzed to form glutamate. Other conditions including temperature (not shown) and duration of crosslinking (Fig. 2B) play a role; these factors should be considered further. Also, if specific products are desired (e.g., dimer, trimer, etc.), various purification methodologies could be added such as size exclusion chromatography or salt precipitation.

In this work, we also demonstrated control of metabolic flux by the addition of rate-limiting LuxS (Fig. 3C). The increased flux obtained from each round of additional crosslinking was apparently linear however and instead appeared to saturate with the number of added proteins

(Fig. 3D and 3E). Our approach was helpful in constructing multi-functional complexes (Fig. 4) and this not only proved beneficial as we changed the orientation of complexes finding resuscitation of enzymatic activity, making them both effective and multifunctional, but also provided insight into the decrease in flux obtained per unit enzyme. That is, since the addition of Protein G and EGFP decreased the activity of LuxS, the rate-limiting factor, we hypothesize that addition of subsequent LuxS subunits may also decrease the preceding LuxS subunit's activity, thereby leading to overall decrease in per unit enzyme activity with each round of crosslinking. It would be interesting to study how these complexes perform in comparison to multiple gene fusions.

The use of Co^{2+} beads provided control over crosslinking orientation and helped create 'deployable' complexes. Since we assembled enzymatic complexes that were involved in generation of QS responses, by controlling the sequence of crosslinking and orientation of the subunits, we could mediate and control small molecule communication between an assembled abiotic complex and cells leading to altered metabolic outcomes. We note this lysine and glutamine tag/transglutaminase methodology can be used to fabricate protein complexes that retain function of each assembled subunit.

In addition to building complexes that have utility in solution, as demonstrated by altering QS communication *in vitro*, we expect this methodology will find utility in assembling biological components in conjunction with cells and tissues in lab-chip or animal-on-a-chip systems. Biological components embedded with electronics can sense, report and modulate the tissues and cells of interest [7]. Developing methodologies to assemble enzyme cascades in these systems would be valuable in providing greater access and even control. Hence we believe that the solid phase synthesis approach can be translated to microfluidic and microsystem environments where

multiple components can be flowed in and immobilized to each other [13], rather than existing techniques that utilize harsh immobilization chemistries, or valves, pumps, and printing methodologies.

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