**Abstract**

Oligomerized hemoglobin molecules have been shown to decrease previously observed deleterious effects from treatment with cell-free hemoglobin. To create these constructs, a method was developed which employs the site specific ligation reaction of the sortase A enzyme from S. aureus. Previously developed Hb mutants (sc-Hb) were further modified by adding either the sc-Hb recognition sequence, LPXTG, to the C-terminus of the α-subunit (s-αcp), or a tetrazine motif to the N-terminus (n-αcp). Three types of sortase mediated ligation (SML) will be employed in this study. First, we will attempt to ligate Hb subunits directly, using a verified functional groups. To begin with, we will be using doubly modified Hb, and so our products will be a mixture of s-αcp (substrate) and n-αcp (nucleophile). The second method will be two-part, first appending an azide or strained cyclooctyne to the C- and N-termini of the subunits. Using the well-established method of Huisgen cycloaddition, the Hb molecules can then be brought together via their chemoselectively appended functional groups. To begin with, we will be using doubly modified Hb, and so our products will be a mixture of oligomeric states. In the future, we will use singly modified monomers to begin and cap our oligomers, as well as alternating the addition of azide of cyclooctyne functionalized subunits to better control the oligomeric states of our products.

**Background: Hb Mutants**

Oligomeric constructs of native Hb have been shown to attenuate the adverse effects of intravenous transfusion of cell free Hb. Here, an octameric Hb construct is shown to attenuate the hypertension response attributed to its decreased ability to permeate through blood vessels and scavange nitric oxide.

**Methods: Hb Modification and Click Handle Synthesis**

![Click handle synthesis diagram](image)

The methods for Hb modification and click handle synthesis involve the use of sortase A to mediate the ligation of functionalized Hb monomers. The reaction conditions include:
- **Terminal modification of GPP**
  - 50 µM L-PAP
  - 100 µM Tris-HCl (pH 7.5)
  - 150 mM NaCl
  - 10 mM MgCl₂
- **C-terminal modification of GPP**
  - 50 µM L-PAP
  - 100 µM Tris-HCl (pH 7.5)
  - 150 mM NaCl
  - 10 mM MgCl₂

**Background: MASML**

Metal assisted sortase mediated ligation has been shown to double yields when ligating synthetic peptides to full sized proteins. The figures to the right depict reactions in which GPP acted as either substrate or nucleophile, and the reconstructed MS peaks showing the increase in product yield when treated with 200 µM Ni²⁺.

**Methods: Functionalizing Hb Monomers**

![Functionalizing Hb monomers diagram](image)

Rational design scheme of a single-chain Hb polypeptide used by this lab. First, circularly permuting the beta globin, such that the termini are at the interface with alpha. Then, linking alpha and beta through their respective GH helix loops (sc-αcpβ). Finally, linking native alpha termini together generates sc-Hb.

**Future Work: Dendrimeric Hb**

![Dendrimeric Hb diagram](image)

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