**Protein and peptide engineering for targeted, intracellular delivery**

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Introduction.Protein nanostructures with tunable chemistries would be extremely useful in a wide variety of applications ranging from biocatalysis to biopharma. Biopharmaceutical products are especially well-posed for impact: drugs such as monoclonal antibodies, peptides, and enzymes are rapidly transforming the global pharmaceutical industry.1 Biologics represented 35% of all new FDA approvals between 2010 and 2016,2 and they accounted for 71% of the revenues from the top ten-selling pharmaceutical products in 2012, *vs.* 7% in 2001.3 Moreover, much of the therapeutic target space remains untapped: for example, the pipeline has a dearth of intracellular protein drug candidates,1 even though intracellular proteins comprise 61% of the proteome and have predicted therapeutic applications ranging from cancer to neurological disorders to lysosomal storage diseases. In fact, essentially all protein drugs to date have been restricted to vascular targets or targets on the cell surface, referred to as the ‘accessible target space.’1

Aims. We are designing protein conjugates and protein/polypeptide nanostructures that will illuminate and enable new aspects in protein encapsulation, transport, and cellular processing. Our specific goals are to demonstrate the use of versatile and modular protein engineering approaches (*e.g.*, ‘Tag/Catcher’ chemistries;4 unnatural amino acid [UAA]/‘click’ chemistries5) to create protein nanostructures that can be targeted and delivered with high efficiency, cell specificity, and no activity loss.

Methods. We have employed Tag/Catcher and UAA insertion strategies as a method to site-specifically conjugate delivery moieties to therapeutic proteins and modified viral capsids (e.g., hepatitis B virus, or HBV). We specifically explored the effect of epidermal growth factor receptor (EGFR)-targeted ligand valency and spacing on internalization of proteins in EGFR-overexpressing inflammatory breast cancer (IBC) cells.

Results and Discussion. Our results demonstrate the ability to enhance targeted protein delivery in IBC cells by tuning the number and density of EGFR ligands, and by altering the size/morphology of the protein nanostructure used for delivery. Furthermore, we demonstrate that our approaches can be easily applied to delivery of a variety of cargoes (e.g., visible proteins; the suicide enzyme yeast cytosine deaminase [yCD], an enzyme that converts the nontoxic prodrug 5-fluorocytosine [5-FC] into the toxic chemotherapy drug 5-fluorouracil [5-FU]). Co-delivery of yCD and 5-FC resulted in significant IBC cell death, with the levels of cell death controllable through alterations in ligand number. Furthermore, nanostructure size and the location of conjugated ligands affected cell specificity, delivery efficiency, and pharmacological activity in proteins, a phenomenon that has not been studied extensively in protein drugs due to conjugation limitations.

**References**

1. Mitragotri S, Burke PA, Langer R. Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. Nature reviews Drug discovery. 2014;13(9):655-672.

2. Biotech products accounted for 35% of all new FDA approvals in 2000-16. Impact Report: Analysis and Insight into Critical Drug Development Issues: Tufts Center for the Study of Drug Development; May/June 2017.

3. Biotech products in Big Pharma clinical pipelines have grown dramatically. Impact Report: Analysis and Insight into Critical Drug Development Issues: Tufts Center for the Study of Drug Development; November/December 2013.

4. Zakeri B, Fierer JO, Celik E, Chittock EC, Schwarz-Linek U, Moy VT, Howarth M. Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. Proceedings of the National Academy of Sciences. 2012;109(12):E690-E697.

5. Wang Q, Parrish AR, Wang L. Expanding the Genetic Code for Biological Studies. Chemistry & Biology. 2009;16(3):323-336.