Small molecule probes for imaging matrix metalloprotease activity Rachel Matt, Stanford

Key words: matrix metalloprotease, integrins, ERK signaling, chemical probe, cell motility

Introduction: How do cells sense their extracellular environment and transduce that information into a signal for cell movement under varying conditions? The answer may come from a subset of the protease enzyme family, the matrix metalloproteases (MMPs), which were traditionally thought to function only as extracellular matrix degrading proteins. However, recent studies have shown more complex functions for MMPs in cell signaling and motility[1,2]. Membrane-bound MMPs are regulated by post-translational mechanisms including proteolytic cleavage, phosphorylation, and internalization-recycling pathways. Thus, it is not possible to perform dynamic studies of their activity by traditional proteomic or genetic methods. A solution is to use synthetic small molecule probes, which confer the spatial and temporal control needed to study activity of enzymes under intricate regulation. I propose to use a small molecule probe that will covalently react with an introduced cysteine residue near an MMP enzyme active site, tracking the active protease inside a cell through fluorescence. By co-engineering the target protein and synthetic probe as a pair, similar to building a custom lock and key, I can attain selective inhibition of the protease while observing sub-cellular localization by fluorescence imaging.

Within the MMP family, matrix metalloprotease – 14 (MMP-14) is credited with degradation of many extracellular matrix components, which clears a path for cell migration. MMP-14 activity also enables cell migration through activation of the extracellular signal-related kinase (ERK) signaling cascade,[3] but the molecular mechanisms for this pathway activation remain unknown[2]. Since both ERK signaling and recycling of MMP-14 through endosomal compartments are critical for endothelial cell motility,[1,2] one possible mode of ERK activation is through MMP-14 endocytosis from the plasma membrane. MMP-14 is endocytosed and recycled with another transmembrane protein, integrin α5. Integrins establish cell adhesion under a variety of conditions, providing another mode for transducing extracellular information. Integrin α5 is cleaved to its active form by MMP-14, and phosphorylation of MMP-14 is required for co-trafficking with the integrin[1]. While integrin α5 cleavage has also been shown to stimulate cell motility[1], its proteolytic cleavage has not yet been directly linked to MMP-14 endocytosis, and the evidence for this coordinated influence on ERK signaling has only been correlational. **I thus hypothesize that cleavage of integrin α5 by active MMP-14 stimulates endosomal recycling and downstream ERK signaling to facilitate wound-induced migration of endothelial cells.**

Specific aim 1: **Synthesize a fluorescently-quenched small molecule MMP-14 inhibitor.** Current MMP-14 inhibitors cross-react with other proteases. I aim to specifically inhibit MMP-14 with a small molecule that covalently binds the protease through a purposefully engineered cysteine residue near the active site. By engineering both the target protease and the chemical "probe" as a covalent pair, a unique interaction is created that allows controlled perturbations of the protease in biological contexts. Matthew Bogyo's lab at Stanford University has already generated a fluorescent probe that selectively inhibits mutant MMP-14. A quenched version of this probe will have decreased nonspecific binding and lower background fluorescence, allowing real-time imaging of active MMP-14 in live cells. During my rotation in the Bogyo lab, I have imaged this cysteine mutant MMP-14 and have begun modifying the existing probe with a quencher group that blocks all fluorescence until the specific covalent reaction with the protease takes place (Fig. 1). This allows us to overcome the temporal limitations of traditional genetic approaches by inhibiting only the post-translationally modified and active protease. If needed, I will improve imaging properties of the probe by synthesizing alternate linker groups, quenchers, or fluorophores, to improve active site binding, delivery, or quantum yield, respectively.

Specific aim 2: **Determine whether integrin cleavage is required for co-trafficking with MMP-14.** If my hypothesis is correct, I expect that integrin and MMP-14 will be internalized in the same endosomal compartments only when integrin is cleaved by MMP-14 under cell migration conditions caused by inducing a wound in a layer of confluent endothelial cells. I will perform live cell imaging on confluent versus plate-scratched endothelial cells using integrin α5 expressed with a GFP tag, which retains activity[1]. I expect to detect cleavage of integrin α5 by my transfected cysteine mutant-MMP-14, which will be shown by gel analysis. In mutant-transfected cells, the quenched probe will inhibit MMP-14 activity, preventing colocalization with integrin α5. Furthermore, I will express integrin α5 as a non-cleavable mutant; if cleavage is required, then a similar reduction in endosome compartment colocalization will be seen.

Specific aim 3: **Determine whether integrin cleavage by MMP-14 activates downstream ERK signaling under wound-induced migration conditions.** I will detect differences in ERK activity using a live cell FRET-based fluorescent reporter[4], which can be imaged in concert with my Cy5 probe. When non-cleavable integrin α5 is expressed, I expect ERK activation to be decreased relative to wild type. I will then observe a live cell migrating after wound induction and titrate increasing concentrations of a non-fluorescent inhibitor into the growth media. I can use the synthetic precursor of the fluorescent probe as a competitive inhibitor for integrin at the active site; I expect a dose-dependent response in ERK signaling. The inhibitor will thus allow us to temporally control MMP-14 inhibition during cell migration, establishing the role for integrin α5 cleavage and MMP-14 co-trafficking in ERK signaling.

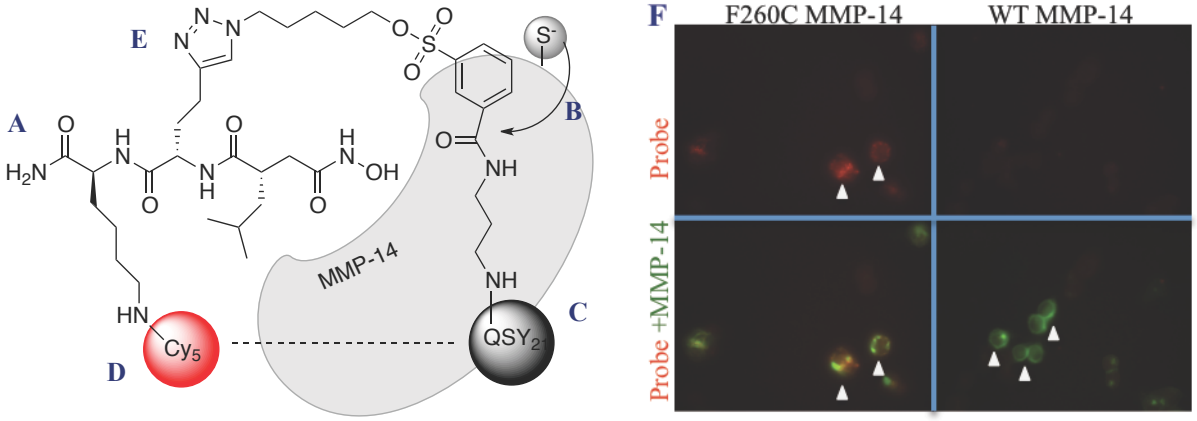

Figure 1: A quenched small molecule probe for specific inhibition and labeling of MMP-14. The peptide-mimetic scaffold **A** positions an electrophilic group **B** for covalent reaction with engineered cysteine residue (F260C mutant) near the active site. Upon reaction, the quencher group **C** leaves, allowing Cy5 group fluorescence, **D**. Synthesis of the probe will proceed easily from an already synthesized non-quenched probe, by attaching the quencher group through a click chemistry reaction at **E**. As proof of concept, the non-quenched probe shows specific labeling of mutant, but not wild type, MMP14 after transfection and fixation of HEK 293T cells, **F**. **Images: Rachel Matt**.

Broader impacts: **My fluorescent chemical probe will reveal a molecular mechanism by which MMP-14 and integrins interact to transduce information about the extracellular environment to an intracellular ERK signaling response for cell migration.** Furthermore, this novel chemical biology approach, which generates a "lock and key" enzyme-probe pair, establishes a generalizable method to assess the functions of proteases. The method will allow detailed characterization of many different proteases in real-time and under near-native tissue conditions, overcoming historical obstacles for studying post-translationally modified enzymes.

1) Williams, K. C. & Coppolino, M. G. *J. Biol. Chem.* (2011). 2) Gingras, D. & Béliveau, R *Biochimica et Biophysica Acta* (2010). 3) Takino, T *Cancer Research* (2004). 4) Harvey, C. D. *Science Signalling* (2008).

Rachel Matt, Stanford University

My passion for sharing scientific information was cultivated underground, during four summers spent giving tours through a dark and damp limestone cave in southeastern Minnesota. As a young and shy high school student, this summer job was invaluable in developing my public speaking skills and in teaching me how to distill complicated scientific concepts to a vacationing public in an educational and interesting manner. During my hour-long walks through the caverns, I engaged hundreds of tourists in learning the rules of that underground world: sedimentation and bedrock geology, fossilization, shifting water tables, and the chemistry of stalactite and stalagmite formation.

The summer tour-guiding job led me on a path to pursue a chemistry major and to educate younger students during my undergraduate career. After falling in love with my chemistry courses, I began tutoring students in organic and analytical chemistry; I was passionate about how a very basic knowledge of organic synthesis allowed me to make predictions about reactions I had never seen before. I loved seeing how my students, after learning these patterns, gained a little more appreciation for a subject that many of them initially dreaded. In a classroom setting, I also taught students in the physics department as a teaching assistant for two years. Here, I gave short presentations about concepts related to those the students were learning in class. For the remaining two-hour class time, I led the students in hands-on experiments to demonstrate the concepts. Teaching my fellow undergraduates reminded me what I had learned during the cave tours: how important it is to complement analogies with visual demonstrations to help make difficult concepts more approachable. Now that I've returned to the academic environment as a graduate student, I'm excited to teach younger students again; I'm especially interested in Stanford's opportunity to teach mini-courses to undergraduate students between academic quarters, since these courses are intended to incorporate the hands-on learning approach that I find so effective. Furthermore, I feel that the methods of my current proposed research, simultaneously co-engineering a protein and its small molecule inhibitor, could be valuable to many of my student peers in their own research; I hope to present my work during our graduate course in Chemical Biology next year, as well as at scientific conferences in the future.

My first opportunities to present my own research also occurred during my undergraduate years. I was fortunate to continue my hands-on approach to learning science in a laboratory. I enjoyed my undergraduate research so much that I wrote a proposal for summer funding through Montana Integrative Learning Experiences for Students (MILES), and was thrilled when I received the award to continue my research for the summer and an additional two semesters. The MILES forums gave me the opportunity to give oral and poster presentations about my chemistry-focused research to an audience specializing in biology. The challenging experience of presenting my work to diverse audiences helped me tremendously in my work after graduation.

After my undergraduate years learning chemistry, biochemistry and mathematics, I sought an opportunity to apply my skills in a work setting. By working in manufacturing and research at GlaxoSmithKline, I saw one example of how basic scientific research could be applied to benefit society. Working on the drug manufacturing line taught me the importance of teamwork in a scientific project, a lesson that I carried with me to a process development role, where I readied a new building for the manufacturing process. I continued teaching my fellow scientists by becoming a qualified trainer for my area of expertise in the manufacturing process. This role was rewarding as well as challenging, as I had to communicate manufacturing concepts and techniques to coworkers who were often much older and more experienced than myself. When I had the opportunity to work with the research group at GSK, developing novel vaccine

adjuvants, my responsibilities shifted to an even greater role in communication. As a member of the formulations research group, I collaborated daily with both medicinal chemists and immunologists. I learned to speak the languages of both disciplines, and I found myself becoming an ambassador between the two groups, able to relay project information between the two since I was working with them daily. I also endeavored to expand the outreach of my formulations group by organizing a chromatography forum that brought together international GSK scientists who worked with analytical chemistry techniques for monthly meetings where we could share our expertise and work together to optimize analytical methods.

While I enjoyed the aspect of industry that focused on deliverable results, optimizing processes, and meeting deadlines, I felt that this outlook meant that scientific results suggesting interesting follow-up biological questions were often ignored, since further investigation would derail the project timeline. I believe this extra investigation into the molecular details of unexpected results would have benefited the project in the long run; for example, by learning more about the physical properties of our vaccine adjuvant formulations, we would be able to manipulate those properties to gain better drug delivery. From experiences such as this, I learned how a better understanding of basic science can benefit, and is necessary for, applied science. This influenced my desire to understand these biological processes at a deeper level and share those insights with those working in related applied fields. However, it was not until I began presenting my research to GSK's international collaborators and took on a leadership role organizing the chromatography forum that I realized I had developed the self-confidence to continue my scientific training as a graduate student. I decided to attend graduate school to further my scientific and leadership training, wanting to learn how to advance basic research that incorporates both chemical and biological techniques.

To apply my skills from industry towards success as a new graduate student, I have found the need to seek advice from my peers and role models in my profession. To accomplish this, I have begun participating in campus-based mentoring programs supported by the Biomedical Association for the Interests of Minority Students, Association for Women in Science, and Women in Science in Engineering. While I am currently receiving valuable advice and support from my peers and mentors in these programs, I hope to give back to the programs as I progress in my graduate career and have formative learning experiences to share with younger students.

I also plan to continue sharing scientific knowledge with nonscientists and across disciplines. In my short time in the graduate program, I have already been able to take advantage of some Stanford's resources for scientific policy and outreach, including a weeklong course in Public Policy and Negotiations. This course allowed me to connect with 20 students in different graduate programs with whom I explored the challenges of educating and presenting technical details from our disciplines to audiences unskilled in our fields. We learned how negotiations techniques and knowledge of bargaining styles could form bridges for effective communication between people of different education and life experiences. The course helped me learn about my own subconscious methods of negotiating, and how can I use these instincts to get my message across more clearly. I will continue to develop my skills in policy and public outreach by participating in a Negotiations and Public Policy club, a forum created by my classmates and myself to allow us to continue learning from one another.

Through these organizations and the extraordinary research opportunities available to me at Stanford, I am sure that I will emerge from the Chemical and Systems Biology program as a confident and societally engaged researcher, capable of sharing my discoveries and shaping their use in both basic and applied fields.

Rachel Matt, Stanford University

My research experiences as an undergraduate and during my employment in the pharmaceutical industry have been at the interface of chemistry and biology. Through my research at Nigel Priestley's lab at the University of Montana and at GlaxoSmithKline Biologicals, I became fascinated by the application of chemical tools towards biological systems.

I was fortunate to begin undergraduate research as a sophomore in a lab focused on bioorganic chemistry. The Priestley lab studies bacterial natural products, and my specific project investigated the polyketide synthase (PKS) biosynthetic pathway of *Saccharopolyspora spinosa.* We attempted to knockout one domain of the PKS pathway in order to produce the bacterium's natural product, spinosyn, with an unreduced double bond incorporated into the structure. The introduced double bond could serve as a chemical handle for further chemical reactions mediated by Grubbs catalysis after the product was isolated by bacterial fermentation and extraction. I worked closely with a graduate student in the lab to optimize fermentation conditions for production of spinosyn, and I learned molecular biology techniques by preparing plasmids with the incorporated genetic modification in the PKS domain. I independently generated an electrocompetent bacterial strain to introduce the recombinant plasmids and just before graduation, began screening colonies for our introduced mutation to generate the modified spinosyn.

We planned to further modify the spinosyn structure using chemical reactions on the introduced double bond to generate a diverse compound library that could be screened for biological activity. We anticipated that a library based on this scaffold could have novel antibiotic properties, since *Sacc. spinosa* is related to *Sacc. erythraea,* which produces the macrolide antibiotic erythromycin through a different PKS pathway. To explore the structural diversity of a library based on this scaffold, I independently generated the spinosyn library virtually, even before synthesis, by encoding the structure in a computational string. I used a principal components analysis to reveal a subset compounds in the potential library that encapsulated the diversity of the entire set. This allowed us to select future target molecules for synthesis using the introduced double bond. Though I graduated from the university before we were able to generate the synthetic library, I presented my results from the project in a written final honors research report and in an undergraduate research poster conference. While I initially I joined the Priestley lab hoping to expand on my interests in organic chemistry, I was surprised to learn computational chemistry and molecular biology techniques along the way; I found that the computational analysis complemented the project well, making the library generation process more efficient. Additionally, I enjoyed learning about polyketide synthesis on a molecular level, finding that an approach to biological systems at the molecular level of chemical reactions was very satisfying to me.

After graduation, I worked for nearly two years in a vaccine adjuvant research group for GlaxoSmithKline Biologicals, a role that built upon my previous laboratory research both scientifically and professionally. Instead of working with graduate students, my supervisors expected me to define my own role in the project and to set my own deadlines. Working in a formulations and drug delivery group, my primary goals were to develop formulations for aqueous delivery of synthetic molecules and to quantify the concentration and stability of these molecules through analytical chemistry techniques. I learned quickly that working in this independent environment required me to be able to troubleshoot problems with laboratory equipment and techniques on my own. After I developed some troubleshooting skills by rebuilding an old HPLC instrument, I was able to work on optimizing the chromatography

methods for our assays. Success in this project gave me the confidence to tackle a larger one; several attempts had been made at improving the water-solubility of one class of vaccine adjuvant candidates. Even though several research group members felt that we would not be able to improve the intrinsic solubility of these molecules, I was willing to give it a try. I researched the current formulations literature and discovered a high throughput method that I felt could be applied to our adjuvants. I began by screening our lead compound in a variety of cosolvent formulations, which resulted in modest solubility improvement. Encouraged, I applied the technique to novel salt forms of the synthetic molecules, one of which improved the solubility of our lead compound over 40-fold. This improvement allowed the compound to be tested in our in vivo model. Throughout this process, I learned how discoveries at the lab bench could be developed into technical advances in the field of formulations. I also learned how to research the literature and synthesize my findings into an experimental design. Yet I think my most important lesson was in perseverance; I learned the rewards of following through on an idea, even when past attempts have been unsuccessful.

During my industry experience, I also learned the benefits of keeping the ultimate goals of the project in mind. I struggled with this mindset with as an undergraduate researcher, where I sometimes became so focused on understanding the intricacies of a particular technique that I ignored the method's usefulness in answering a larger question. The pharmaceutical industry aim to develop a 'drug pipeline' kept me constantly reminded that the broader goal of my project was to evaluate the therapeutic potential of our vaccine adjuvant candidates. Understanding the impact of my laboratory work gave me direction in my daily tasks. For example, after unusual in vitro results from one experiment, I tested the active compound to determine whether a difference in synthetic chemistry could be the cause. By following up, I discovered a significant variation in the physical properties of the material over time. Because of a solid understanding of the ultimate goals of our project, I was able to see the implications for other adjuvant candidates, so I brought together a group of chemists and immunologists to determine how we could apply the findings. We quickly decided to change the aqueous formulation for improved stability, keeping the project on track. Thus my shifted perspective towards the project's ultimate goal me more helpful in advancing the project through the preclinical phase, and allowed me to better communicate the project's needs with my colleagues in other disciplines. I continued to expand my scientific communication skills by presenting my formulations work to collaborators both locally and internationally during monthly oral presentations lasting 30 minutes to one hour. Working with such a large and diverse group of researchers made me excited to continue such collaborations in my graduate career.

I began my graduate studies at Stanford University in July with an early summer laboratory rotation under Daria Mochly-Rosen. I used a peptide inhibitor to disrupt protein-protein interactions between a mitochondrial outer membrane protein and its docking partner. By perturbing this interaction, I was able to characterize the importance of the interaction in mitochondrial morphology, and consequently, in mitochondrial energy production. My second rotation is a more chemistry-focused project with Matthew Bogyo, whose synthetic inhibitors of proteases have inspired me to understand the function of matrix metalloproteases in signaling and cell motility. I feel that both of these laboratories have allowed me to pursue my interests in both chemistry and biology. I intend to continue developing chemical tools that can be to specifically inhibit cell biological interactions, giving insight into their importance and function. Furthermore, I plan to share the new tools I develop with the broader scientific community through publications and presentations, expanding their utility to other biological problems.

Score for Matt, Rachel

Intellectual Merit Criterion

Overall Assessment of Intellectual Merit

Excellent

Explanation to Applicant

The applicant does not have any publications or national poster presentations, but has garnered multiple fellowships and academic awards. One award led to presentations. The applicant has worked as a research scientist as GSK for almost 3 years. Forums were organized at GSK to enhance communication across the company and these demonstrate leadership. Experience at GSK has given this applicant research experiences that is unique for students at this level. The research is well communicated for both the past and future work.

Broader Impacts Criterion

Overall Assessment of Broader Impacts

Very Good

Explanation to Applicant

The applicant has experience as a cave tour guide, and an undergrad tutor and TA. The applicant has taken part in mentoring programs and plans to give back to these programs in the future. The broader impacts of the past research and the future research are well described. This proposal would be strengthened by clear past and future plans of scientific outreach.

Score for Matt, Rachel

Intellectual Merit Criterion

Overall Assessment of Intellectual Merit

Excellent

Explanation to Applicant

The applicant's undergraduate academic performance was excellent. The applicant became matured through the work experience at the biopharmaceutical company. The reference letters and the numerous recognitions strongly indicate that the applicant has strong intellect and great communication skills.

Broader Impacts Criterion

Overall Assessment of Broader Impacts

Very Good

Explanation to Applicant

The applicant served as a teaching assistant and a tutor at multiple times. The applicant itself received the benefits from the mentoring programs designed for the underrepresented groups, and has a plan to participate in those programs in return. The social impacts of the proposed research is also discussed.

Score for Matt, Rachel

Intellectual Merit Criterion

Overall Assessment of Intellectual Merit

Very Good

Explanation to Applicant

The applicant has a strong history of research, both as an undergraduate and as a research technician in industry. Her work has resulted in numerous awards and presentations, but no publication. Her proposal could be strengthened by organizing the aims around specific, testable hypotheses or questions rather than action-driven aims.

Broader Impacts Criterion

Overall Assessment of Broader Impacts

Good

Explanation to Applicant

The applicant proposes to increase the broader impacts of her research by becoming involved in several mentoring programs that are sponsored by the university. She will also continue to hone her communication skills by participating in programs designed to improve science advocacy in the political arena. The applicant could improve her proposal by describing a specific mechanism by which her participation in these programs will increase inclusion of under-represented groups in STEM fields.