**Engineering Ionic Hydrogels to Overcome Charge and Size Barriers in Multi-Protein**

**Co-Delivery Grant Proposal**

Wheatley, Samantha
Massachusetts Academy of Math and Science at WPI
Worcester, MA 01605

**Abstract**

Hydrogels are increasingly being used in biomedical engineering for controlled delivery of chemokines and other therapeutic proteins, yet their ability to release multiple signaling molecules simultaneously remains poorly understood. Individual protein-hydrogel interactions have been widely studied, but co-delivery introduces the additional complexities of intermolecular competition, charge-based interaction, and altered diffusion behavior. These factors are important for the accurate mimicking of immune environments, where cells rely on the coordination of cytokines and chemokines rather than the signals of a single molecule.

This project aims to investigate how the charge of a hydrogel can regulate the simultaneous release of two model proteins with distinct molecular characteristics. The proteins of interest are lysozyme and bovine serum albumin (BSA). Alginate and chitosan are the hydrogels that will be used to observe how the electrostatic interactions influence the release kinetics, protein interaction, and retention during co-delivery.

The study will first establish baseline release behaviors for each protein individually and have this data for future comparison.

*Keywords:* hydrogels, co-delivery, alginate, chitosan, lysozyme, BSA, protein release, protein mimics

## Engineering Ionic Hydrogels to Overcome Charge and Size Barriers in Multi-Protein Co-Delivery

In recent years, hydrogels have gained significant attention in bioengineering due to their ability to mimic the biological environments of the body and support controlled delivery models. Hydrogels are soft, water-rich polymer networks that can resemble natural tissue in mechanical properties, making them valuable in drug delivery, immune-engineering, and regenerative medicine (Peppas & Huang, 2004; Zulfiqar et al., 2022). A common treatment they are increasingly exploring for more often is for osteoarthritis and joint pain, as they can administer drugs directly to the damaged tissue (Grässel & Muschter, 2020). Recent studies have shown that injectable hydrogels can reduce inflammation and improve joint mobility in osteoarthritis models, proving they could potentially be used as a local delivery system (Gan et al., 2024). These findings highlight the growing importance of hydrogel design, as they are capable of achieving a highly controlled release that can study behaviors in biological systems.

### Challenges in Immune-Mimicking Biomaterials

The main challenge of developing biomaterials for immune environments is understanding how the material is going to interact with its surroundings (Hoare & Kohane, 2008). In an immune environment, the interactions can change the outcome of inflammation, regeneration, shaping, and many other processes, making the composition very fragile and complex (Mariani et al., 2019). Material properties such as charge, stiffness, crosslinking density, and polymer chemistry affect how immune signaling occurs. Even though hydrogels have been thoroughly researched and tested in terms of mechanical and chemical properties and interaction, their immunological behavior, especially how the delivery of multiple immune proteins is regulated, is still not thoroughly investigated. Even a small variation in hydrogel chemistry has been shown to affect immune behavior, such as T-cell activity and cytokine expression. This emphasized how immune responses to biomaterials are highly sensitive (Zhang et al., 2022). To better understand how the transport dynamics function, visualizing chemokine gradients within hydrogel matrices can provide insight. These visualizations highlight the complexity of achieving controlled, multi-protein release in engineered systems, which provides a reference for the function of the strategy of the co-delivery.

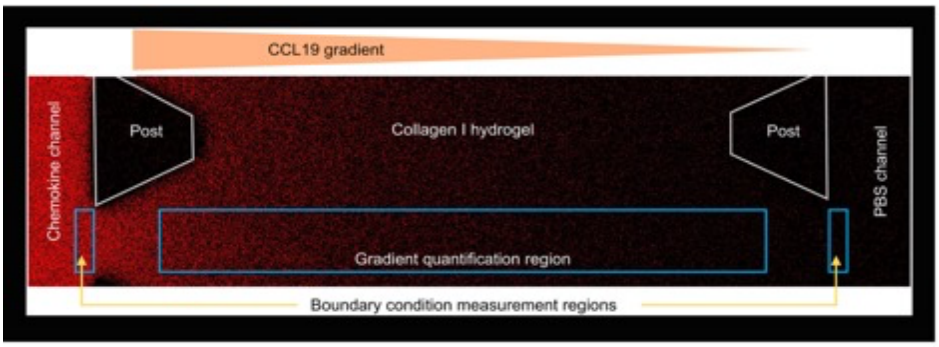


Figure 1. Confocal microscopy scan of steady-state AF647 CCL19 fluorescence in a hydrogel, showing advection opposing diffusion. Chemokine source concentration is 10 nM; magnification is 10× (Bonneuil et al., 2022).

**Importance of Multi-protein Co-Delivery**

Immune signaling in tissue involves more than one molecule, but that is difficult to replicate out of the body. In previous testing, there has been the use of a single molecule that has been proven to be successful, but gradients of multiple proteins guide immune cells through the body. And in an immune model, chemokines and cytokines are vital. This leaves a critical knowledge gap in the co-delivery system, where multiple proteins are released together. These models are much less understood, even with their purpose being essential for a functioning immune signaling environment (Li & Mooney, 2016). The interaction between materials and proteins determines the behavior of the release, and co-delivery differs from that of single release, as the protein interaction, competition, and intermolecular forces will be affected.

**Model Proteins and Hydrogel Selection**

Model proteins are typically used in release studies as they are stable but can still mimic the biochemical properties of typical immune signaling molecules. In this project, the plan is to use two model proteins, lysozyme and bovine serum albumin (BSA). Lysozyme is a positively charged and has a low molecular weight. BSA is negatively charged with a larger molecular weight. The pairing that has been chosen allows for the investigation of how charge and molecular weight affect protein release in hydrogels with different chemistries. BSA and lysozyme have been used in numerous release studies due to their well-documented behavior; this makes them ideal replacements for chemokines when testing biomaterials.

For hydrogels, alginate and chitosan will be used. Alginate is negatively charged, and chitosan is positively charged, allowing for the exploration of the role of electrostatic interaction in protein transportation (Brown et al., 2020). This interaction is expected to influence the retention and release kinetics of proteins, which need to be known to create an effective gel. It is hypothesized that lysozyme will release more slowly in the negatively charged alginate and more rapidly in the positively charged chitosan. BSA is projected to behave in an opposite manner, which it will release more slowly from the positively charged chitosan and more rapidly in the negatively charged alginate. Initial experiments will be done to quantify the release of the individual proteins to establish a baseline of their behaviors. Once these are conducted, they will be followed by the co-delivery studies to determine how the dual protein interactions will influence the release.

**Research Objectives**

The overall goal of this work is to establish a foundation of understanding in how the charge and mechanics of a hydrogel can regulate protein release. In comparing single and dual protein release, this will generate essential baseline data for the initial design of biomaterials capable of mimicking more complex immune signaling environments. These findings and new knowledge will support future development in immune-mimicking hydrogel systems in bioengineering applications.

## Section II: Specific Aims

This proposal's objective is to determine how hydrogel charge influences the simultaneous release of proteins and to provide insight into how co-delivery systems interact with immune biomaterials.

The long-term goal is to engineer biomaterials that are capable of mimicking the complex environment surrounding immune signaling by delivering multiple model chemokines with control and precision. The central hypothesis of this proposal is that hydrogel charge and chemistry will play a critical role in the regulation of the individual and co-delivery of protein. The parameters of the gels will be tuned to achieve a balanced release profile. The rationale is that understanding the fundamental interactions between the proteins and hydrogels individually will translate to the predictions of the co-delivery. Understanding how the protein's charge, molecular weight, and chemistry of the hydrogel are essential for designing an effective and efficient co-delivery system. The work we propose here will create the foundational data that shows how dual-release systems behave and how a hydrogel can be properly engineered to achieve predictable release kinetics for multiple molecules.
**Specific Aim 1:** Quantify the effect of hydrogel charge on the individual release of the model proteins, lysozyme and BSA.
**Specific Aim 2:** Determine how co-delivery affects the release kinetics of proteins with different charges and molecular weights.
**Specific Aim 3:** Relate hydrogel properties, such as charge and polymer type, to the release behavior of the proteins to develop basic design rules for immune-mimicking biomaterials.
The expected outcome of this work is an understanding of how alginate and chitosan hydrogels can regulate the release of proteins individually and together. This will provide the fundamental design outline for future immune modeling.

## Section III: Project Goals and Methodology

### Relevance/Significance

This project addresses a critical knowledge gap in the understanding of how material properties control the release and co-delivery of chemokines. As this project focuses on co-delivery, it addresses the difficulty of releasing multiple signaling molecules simultaneously and at balanced rates. This specific type of control is important, especially when mimicking the immune environment and designing better biomaterials that can coordinate multiple cell signals. This research will provide foundational insight that aims to support future applications in immunotherapy and regenerative medicine, as precise control of chemokine signals is crucial for success.

### Innovation

There are numerous innovative aspects of this project. Some of them are the choice of hydrogel and proteins, the experimental structure, the application of model proteins, and the direct comparison between releases. The use of oppositely charged hydrogels and proteins is used to understand how electrostatic interactions determine release. The experimental structure was a single release, is tested first to get a baseline moved into co-delivery, isolating the effect protein competition will have on the results. The use of model proteins allows for this project to be safe and reproducible, allowing the results to be applicable to more complex signaling proteins in future work, while not having to take risks early in the research. The direct comparison between the individual and simultaneous release is rarely studied but is crucial for understanding real immune environments and how this process will be expressed within a human.

### Methodology:

Before I begin the trials with lysozyme and BSA, I am using FITC dextran to test the integrity of the release of each type of hydrogel. FITC is used as it allows for the release to be easily measured using a fluorescent plate reader.

***Specific Aim #1:***

Determine the effect of hydrogel charge influences the individual release of lysozyme and BSA.

**Objective.** The objective is to quantify how hydrogels with different charges affect the release behavior for each protein. Our approach uses positively and negatively charged hydrogels and release assays to create cumulative release curves.

**Justification and Feasibility.** Hydrogel-protein interactions are known to depend heavily on charge due to their electrostatics. Prior work has shown that positively charged hydrogels slow the release of negatively charged proteins due to their electrostatic attraction. This supports the hypothesis that lysozyme, a positively charged protein, and BSA, a will interact with each hydrogel differently based on the charge associated with their type.

**Summary of Preliminary Data.**

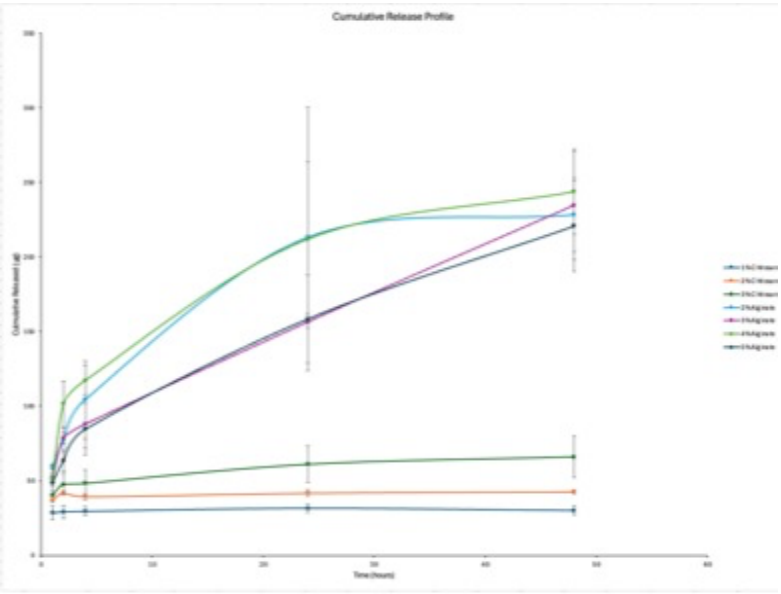


Figure 2. Compiled FITC-dextran release over 72 hours from alginate and chitosan hydrogels with different concentrations

The initial testing with the FITC-Dextran acts as a predictability model for the diffusion of the alginate and chitosan over 72 hours (Figure 2). These results confirm that the hydrogel preparation is reliable and that the cumulative release curves can be made quickly and accurately.

**Expected Outcomes.** We expect that each protein will follow a predictable charge-dependent trend. Lysozyme is expected to release more slowly from the negatively charged hydrogel, while BSA will release quickly. But BSA will be retained within the positively charged gels longer, and lysozyme will escape faster. This information will be used to establish a baseline in understanding for the interpretation of co-delivery behavior seen in the second aim.

**Potential Pitfalls and Alternative Strategies.** We expect minimal issues with this strategy, but if the charge does not have as great an effect as we originally projected, we can increase the charge density or adjust the hydrogel crosslinking to enhance the interaction between the protein and the matrix. If the protein adsorption ends up interfering with the release measurement, we will quantify the surface for binding separately and correct the release curves we got originally.

***Specific Aim #2:***

Determine how co-delivery affects the release kinetics of proteins with different charges and molecular weights.

**Objective**

**Justification and Feasibility.**

**Summary of Preliminary Data.**

**Expected Outcomes.**

***Specific Aim #3:***

Relate hydrogel properties, such as charge and polymer type, to the release behavior of the proteins to develop basic design rules for immune-mimicking biomaterials.

**Section III: Resources/Equipment**

**Section V: Ethical Considerations**

**Section VI: Timeline**

**Section VII: Appendix**

**Section VIII: References**

Bonneuil, W. V., Watson, D. J., Frattolin, J., Russell, M. J., Fasanella Masci, F., Bandara, M., Brook, B. S., Nibbs, R. J. B., & Moore, J. E. Jr. (2022). Generation of stable advective-diffusive chemokine gradients in a three-dimensional hydrogel. *AIP Advances, 12(2), 025121.* https://doi.org/10.1063/5.0064947

Brown, T. D., White, J. J., & Khan, S. A. (2020). Evaluating dextran-based probes for hydrogel transport studies. *Biomaterials Science, 8(12), 3345–3355.* https://doi.org/10.1039/D0BM00543A

Gan, X., Wang, X., Huang, Y., Li, G., & Kang, H. (2024). Applications of Hydrogels in Osteoarthritis Treatment. *Biomedicines, 12*(4), 923. https://doi.org/10.3390/biomedicines12040923

Grässel, S., & Muschter, D. (2020). Recent advances in the treatment of osteoarthritis. *F1000Research, 9(F1000 Faculty Rev), 325.* https://doi.org/10.12688/f1000research.22115.1

Hoare, T. R., & Kohane, D. S. (2008). Hydrogels in drug delivery: Progress and challenges. *Polymer, 49(8), 1993–2007.* https://doi.org/10.1016/j.polymer.2008.01.027

Li, J., & Mooney, D. J. (2016). Designing hydrogels for controlled drug delivery. *Nature Reviews Materials, 1(12), 16071.* https://doi.org/10.1038/natrevmats.2016.71

Mariani, E., Lisignoli, G., Borzì, R. M., & Pulsatelli, L. (2019). Biomaterials: Foreign bodies or tuners for the immune response. *International Journal of Molecular Sciences, 20(3), 636.* https://doi.org/10.3390/ijms20030636

Peppas, N. A., & Huang, Y. (2004). Hydrogels and drug delivery. *Advanced Drug Delivery Reviews, 56(11), 1531–1532.* https://doi.org/10.1016/j.addr.2003.10.015

Zhang, D., Li, Q., Chen, X., Nie, X., Xue, F., Xu, W., & Luan, Y. (2022). An injectable hydrogel to modulate T cells for cancer immunotherapy. *Small, 18*(32). https://doi.org/10.1002/smll.202202663

Zulfiqar, M., Lee, S., & Kim, S. H. (2022). Hydrogels for protein delivery in biomedical applications. *Journal of Controlled Release, 343, 598–613.* https://doi.org/10.1016/j.jconrel.2022.01.015