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ENGS 89/90 Final Report
6 March 2020

Amino Acid Quantification for DMC Biotechnologies
DMC Biotechnologies

Group 26.420

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Acronyms Defined

AAA Kit	Amino Acid Quantification Kit from CellBiolabs
AuNP	Gold Nanoparticles
DMC	Dynamic Metabolic Control; Sponsoring Company
FTIR	Fourier-transform infrared spectroscopy
MIP	Molecular Imprinted Polymer
MIP-NAA	Molecular Imprinted Polymer coupled with Nanoparticle Agglomeration Assay
NAA	Nanoparticle Agglomeration Assay
NIP	Non-imprinted Polymer
NPV	Net Present Value
SEM	Scanning Electron Microscope
SOA	State-of-the-Art
UPLC	Ultra-Performance Liquid Chromatography
YPD	Yeast Peptone Dextrose

Executive Summary

Significance: From pharmaceuticals to biofuels, fermentation bioprocessing has gained traction in a variety of industries, allowing the sector to reach a global market value of almost \$60 billion in 2018.¹ The perceived advantages of replacing non-renewable petroleum-based hydrocarbon feedstocks with renewable biomass feedstocks have made bioprocesses an attractive alternative to traditional chemical processes.^{2,3} However, applications of bioprocessing have been limited in scope due to high capital costs, long development times, and difficulties maintaining productivity during scale-up. The team's sponsor, DMC Biotechnologies, has created a platform that reduces the cost and time of strain-development, which has allowed the company to enter into small-volume markets while maintaining a return on investment. After developing a bacterial strain, DMC's bioprocesses scale-up relies heavily on the monitoring and control of the bioreactor system inputs and outputs. Currently, state-of-the-art analytical chemistry techniques used by DMC, such as ultra-performance liquid chromatography (UPLC), are low-throughput, have high fixed and variable costs, and require complex method development for each new analyte of interest, impeding experimental analysis for process scale-up.

Problem Statement: The development and scale-up of fermentation bioprocesses require careful monitoring and control through analytical chemistry. Currently, there are no small molecule quantification techniques that are accurate, inexpensive, and high-throughput.

Innovation: The team conceived and advanced towards a novel quantification assay in the form of a two-part amino acid sensor. Using molecularly imprinted polymer (MIP) technology in conjunction with gold nanoparticle (AuNP) agglomeration, progress was made towards developing a comprehensive MIP microplate to be used as a selective filter for amino acid separation and sensing. A protocol was devised for reliable MIP manufacturing in a 96-well plate to ensure that microplates have desirable thickness, porosity, and uniformity. As chemical interactions with AuNPs yield a plasmonic signal, a colorimetric assay was developed for the quantification of the analyte of interest, leucine, and its structural isomer, isoleucine. These assays repeatedly showed a linear plasmonic response with concentrations between 0.005 and 1.05 g/L with an R² value greater than 0.95.

Approach: The final assay is composed of three primary elements: a nylon-6-based MIP recognition material, an optical transduction mechanism, and a signal analysis system. After developing a MIP for specific amino acid capture, the team shifted its focus to the transduction element of the assay by attempting to incorporate synthesized gold nanoparticles (AuNPs) into the nylon-6-based MIP. Analyte capture was hypothesized to cause swelling in the polymer, increasing the distance between AuNPs and changing the location of their peak absorbance. However, the absorbance spectra of MIPs-AuNP composites proved difficult to analyze and were highly dependent on surface uniformity. As such, the team decoupled the analyte capture from the transduction mechanism by changing the configuration of the assay to be: 1) analyte capture in MIP, 2) analyte release into AuNP sensing solution, causing agglomeration of the AuNPs, and 3) absorbance redshift measured on the spectrometer.

The team's experimentation proved that the synthesized AuNPs agglomerate predictably in the presence of aqueous analyte, which has been used to repeatedly construct a linear response-versus-concentration curve across different batches of AuNPs. Comprehensive testing of the decoupled assay components against the KPIs of specificity, durability, and sensitivity in the presence of fermentation broth species showed consistently promising results. The team will deliver to the sponsor a two-pronged tech transfer package comprising: 1) a nanoparticle agglomeration assay (NAA) that can be immediately integrated into their chromatography workflow and 2) a significantly progressed MIP-based small molecule quantification component that, once refined in the presence of DMC's fermentation broth, can be coupled with the NAA and integrated into the sponsor's workflow in the near future. The sponsor will be provided with detailed documentation of the team's research, experimentation, finalized protocols, and MATLAB code to aid the tech transfer and outline a path toward attaining the desired assay specificity in DMC's fermentation broth.

Impact: A quick and efficient small molecule assay will allow DMC to more rapidly assess the performance of fermentation processes. The technology developed over the course of ENGS 89/90 will bolster DMC's position in small-volume markets by reducing R&D overhead. Rapid performance assessment is critical for DMC to continue diversifying their product pipeline and progress fermentation as a potentially more eco-friendly alternative to traditional chemicals manufacturing.

1. Problem Statement and Significance

From cosmetics and food to cleaning products and clothes, consumer chemicals form an integral part of everyday life. In 2017, the global chemicals market attained revenues greater than four trillion dollars, but traditional industry practices rely on nonrenewable, petroleum-based hydrocarbon feedstocks. Fermentation chemical manufacturing, or bioprocessing, presents a potentially more sustainable alternative by leveraging microbial biocatalysts in the conversion of conventional sources of carbon, such as raw sugarcane, wheat, and corn, to products. Crucial to fermentation development are microbial strain selection and optimization, media and process development, and scale-up to maximize efficiency. Although fermentation bioprocesses have been adopted in the production of nutritional ingredients and pharmaceuticals, significant challenges in scale-up and development have hindered more widespread commercial success. Existing scale-up strategies are resource-intensive, requiring a high degree of monitoring and control, making them incompatible with the biotechnology industry's rapid growth, wide scope, and sophisticated operations.

The sponsor of this project, DMC Biotechnologies, is an innovator in the fermentation market due to their significantly lower time and cost of bacterial strain development. With the company's efficient metabolic engineering platform, DMC can pursue multiple small-volume markets while maintaining a return on investment. DMC finds its place among startups like Culture Biosciences, Gevo, and Manus Bio that are working to improve metabolic engineering of microbes for more efficient bioprocesses that reach higher yields. DMC's technology aims to reduce biological complexity and enhance the speed of development, creating a low-cost, fermentation-based manufacturing platform that can produce a variety of products. After a bacterial strain is developed, the scale-up of DMC's bioprocess relies on the monitoring and control of bioreactor system inputs and outputs. Currently, DMC uses ultra-performance liquid chromatography (UPLC) to assess the productivity of fermentation operations. A form of column chromatography, UPLC is the gold standard for separating, identifying, and quantifying carbon compounds. However, this technique is low-throughput, has high fixed and variable costs, and requires complex method development for each new analyte of interest, impeding experimental analysis for commercial production levels. Additionally, biologics-based, high-throughput assay technologies, such as coupled enzyme and immunosorbent assays, are costly, unspecific, and are often incompatible with fermentation broth conditions. These disadvantages make UPLC and biologics-based assays insufficient for DMC's long-term objectives. Consequently, the widespread implementation of fermentation bioprocesses is limited by the absence of small molecule quantification techniques that are accurate, inexpensive, and high-throughput.

2. Technical Objectives

This project aimed to develop a low-cost, high-throughput small-molecule detection platform capable of accurately quantifying a variety of analytes. The team built on existing molecular imprinted polymer (MIP) literature that cites MIPs as highly specific small-molecule detection material. DMC conducted preliminary research into potential technologies and isolated MIPs because of their reported robustness, specificity, and low manufacturing cost.^{4,5} These polymers have been successfully implemented as accurate small-molecule sensors in research studies and as commercial products.^{6,7,8,9,10} DMC is interested in developing 96-well analytical assays based on this technology to better monitor and control their fermentation processes during development and scale-up.

This project's experimental objective was to build on DMC's preliminary MIP investigations for the specific capture of two candidate amino acids, leucine and isoleucine. The team proposed a solution that would economically scale a MIP quantification method, incorporating both analyte detection and transduction methods based on reported laboratory success.^{11,12,13} The questions this study sought to answer are as follows:

1. What MIP composition will lend a highly sensitive and specific recognition material for amino acids in aqueous solutions?
2. How can transduction be optimized to translate analyte capture in the MIP into a linear, optical signal on the spectrometer?
3. How might the development and integration of this assay platform be implemented in a sufficiently cost-effective and time-saving manner?

Project specifications were adapted from solution requirements outlined by DMC and the current literature. In order of importance, the specifications are cost, accuracy, repeatability, dynamic range, specificity, robustness, adaptability, and time-to-result. As DMC diversifies their product pipeline, they must increase the number of weekly experiments their R&D department can conduct. Using current company protocol, this scale-up would require purchasing additional UPLC machines, which cost between \$50,000 and \$60,000 used from LabX.com, and hiring more operation technicians, an occupation that Glassdoor.com cites with an average annual salary of \$55,000. Additionally, variable material costs range from \$1,180 to \$1,850 per 1000 UPLC samples due to the price of high-grade reagents, plates and vials, and chromatography columns (Supplementary Materials 1. Head-to-Head Breakdown). Preliminary MIP investigations indicate that a fixed capital cost less than \$5,000 and a variable cost less than \$1 per sample are feasible (Supplementary Materials 1. Head-to-Head Breakdown).

To meet analytical chemistry standards, MIPs must be within a maximum tolerable error of $\pm 10\%$. Quantification results must be repeatable within a relative standard deviation of less than $\pm 10\%$ across different plates, analysts, and labs. Literature focused on aqueous MIP sensors suggests this level of accuracy and repeatability is feasible.^{14,15,16,17,18,19,20,21,22,23,24,25,26} UPLC can quantify most of DMC's analytes within the range of 0.1 to 5.0 g/L. Most MIP sensors achieve sensitivities within three orders of magnitude around the micromolar to nanomolar range, and the concentration of the template molecule during synthesis has been shown to dictate the effective range.^{27,28,29,30} Therefore, a MIP platform can achieve this range through modification of polymer composition and sample dilution. Lastly, fermentation broth often contains interfering molecules such as cell debris, sugars, ammonium, organic acids, and competing products. The developed platform should identify the analyte of interest with at least ten times greater signal than any of the interfering molecules. Several papers have shown high MIP selectivity of amino acids, even between enantiomers.^{31,32,33,34,35}

Finally, the repeatability, adaptability, and time-to-result of the method will allow the system to be high-throughput. Given the ability to do so, DMC would ideally process 1000 experimental samples per week. This sample volume currently takes two to three weeks on the UPLC. Kupai et. al. have shown that MIPs can achieve more than a hundred binding and unbinding cycles when comprised of the right cross-linkers.³⁶ Therefore, plate reusability might be assessed in the future to further reduce costs. DMC also requires the platform be capable of taking a measurement efficiently, and most MIP-based platforms provide relatively quick responses compared to state-of-the-art (SOA) analytical chemistry techniques.^{37,38,39,40,41} Adaptability will be assessed by the protocol's efficacy across multiple analytes in DMC's pipeline. Time-to-result

will be judged by the ability of a technician to manufacture and obtain accurate results from an assay with less than two hours of hands-on time. These estimates are based on the preliminary experiments run by DMC as outlined in Supplementary Materials 2. Limonene MIP Procedure.

Specifically, the aims of this project include 1) developing a MIP for the selective capture of the team's amino acid product, 2) establishing an optical method of transducing binding into a linear, measurable signal analyzable through MATLAB, and 3) testing and optimizing assay implementation to ensure the prototype meets the sponsor's specifications.

3. Innovation

To facilitate low cost, high-throughput experimentation, the team conceived of and progressed a novel quantification assay in the form of a two-part amino acid capture mechanism using MIP technology in conjunction with gold nanoparticle (AuNP) agglomeration to quantify analyte capture. MIPs are known to be highly specific, making them desirable for small molecule quantification. The team devised a protocol for reliable MIP manufacturing in a 96-well plate with preferable thickness, porosity, and uniformity for high-throughput amino acid capture from test samples. In preliminary experiments, these MIPs demonstrated high capture efficiency and specificity for the analyte of interest. They also showed a linear relationship between sample concentration in the microplate wells and the metric developed to analyze peak shifting in the absorption spectra. Ideally, the sample concentration and the extracted/resorbed amino acid concentrations agree, although some analyte loss is expected throughout the protocol. The team advanced towards using the MIP microplate as a selective filter for amino acid separation and sensing. Optimization of washing protocols for amino acid capture from DMC fermentation broth was attempted but should be investigated further for full-scale implementation.

Additionally, the team developed a colorimetric assay to quantify leucine, the analyte of choice, and isoleucine, its structural isomer, based on chemical interactions with AuNPs yielding a plasmonic signal. The team proposes that this assay functions based on the capability of amino acids to act as electron donors in the reduction of gold (III) chloride and subsequent particle agglomeration as described by the literature.^{42,43} The team coined this method "Nanoparticle Agglomeration Assay," or NAA. These assays repeatedly showed a linear response in the absorption metric with in-well concentrations between 0.005 and 1.05 g/L with an R² value greater than 0.95. Notably, the team demonstrated that this mechanism is relatively robust in the presence of glucose and ammonium, common broth components, but is rendered ineffective with the introduction of carboxylic acids. Therefore, the team proposes the coupling of the NAA with the developed MIP adsorbent in an effort to leverage MIP specificity, which isolates analyte from a majority of the interfering molecules in broth samples. The team also postulates that the NAA can be used as described independent of the MIP to analyze fragments from ion-exclusion chromatography in DMC's workflow given their high amino acid purity.

Combined, the MIP-NAA comprises an amino acid capture and sensing system that has shown the potential to be specific, efficient, cost-effective, and reproducible in fermentation broth. These tools, thus, have implications for the rapid advancement of DMC's research and development, as they would allow engineers to monitor bioreactor production, assess yields of downstream recovery processes, and perform preliminary testing on product quality.

4. Assay Development

4.1 Overview of the MIP-NAA Workflow

The proposed assay workflow consists of three steps that correspond with the three integral sensor components: analyte capture, transduction of analyte capture into a measurable signal, and

signal analysis to derive a sample concentration. First, MIPs are synthesized in the plate wells with an amino acid as the template molecule followed by washing the template out with water (Figure 1.1). A hydrophobic coating was also experimented with to prevent larger proteins in the fermentation broth from sticking to the polymer. Sample broth is added to each well and allowed to sit while the amino acid diffuses into the recognition sites in the MIP. The broth is removed, and water is added to extract the amino acid from the MIP. Next, the NAA is used to transduce the amount of leucine extracted into a readable signal (Figure 1.2). AuNPs are added, which agglomerate in the

presence of the amino acid, causing a redshift in the solution (i.e. greater [Leucine], greater agglomeration, greater absorbance of red light). These unknown samples are typically run in parallel with a set of standard, known-concentration solutions. Finally, the assay is analyzed using a spectrometer plate reader, measuring absorbance at $\lambda = 570$ nm and 460 nm. The data is exported and analyzed using a custom MATLAB script which creates a standard curve, projects the unknowns onto this standard curve, and outputs the estimated concentration for the standards as well as confidence intervals for the estimates.

4.2 MIP Synthesis

4.2.1 Polymer Composition

Designing the MIP required choosing a monomer and solvent that best suited the amino acid template molecule of interest, leucine. Polymer research prioritized proven effectiveness capturing amino acids in aqueous solutions, low cost, and simple, nontoxic preparation. The team pulled from MIP recipes that were thoroughly reviewed, highlighting promising monomers, cross-linkers, solvents, and initiators in amino acid analysis. During ENGS 89, the team investigated two MIP recipes, one based on methyl methacrylate (MMA), and the other based on nylon-6.^{44,45} The team experienced difficulties dissolving MMA and leucine in the same solvent, even after trying various combinations of water, tetrahydrofuran, dimethylformamide, ethanol, methanol, and acetic acid. In contrast, the leucine template was easily dissolved in a nylon-6 and formic acid solution. Given the extensive literature on nylon-6 MIPs for amino acid detection, their ease of synthesis, and preliminary experimental successes with the polymer, the team narrowed the project scope in ENGS 90 to nylon-6-based MIPs.⁴⁶

The phase-inversion method of nylon-6-based MIP synthesis was used based on conversations with and published papers authored by Professor Joseph BelBruno. The protocol involves dissolving nylon-6 and the leucine template in formic acid for 24 hours, pipetting the polymerization mixture into 96-well plates, and allowing the solvent to evaporate in the fume hood overnight.^{47,48} It is hypothesized that the intermolecular hydrogen bonds in the templated polymer network effectively promote the creation of recognition sites.⁴⁹ Evaporation of the formic acid solvent results in an amorphous nylon polymer composed of a “network of amino acid- and self-linked nylon,” as described by Sneshkoff, et. al. The template is extracted with water or 10% aqueous acetic acid, leaving behind precise recognition sites in the polymer that can resorb the template. Once functionalized, nylon-6 serves as a highly specific MIP capture

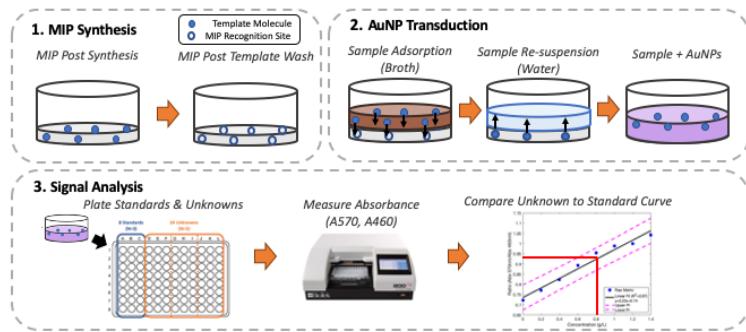


Figure 1 Overview of MIP-NAA workflow: (1) Synthesis of MIPs for analyte capture, (2) Transduction of analyte capture into measurable signal using AuNPs, (3) Signal analysis to determine the concentration of the analyte in the original solution

material. Furthermore, according to the literature, the polymer is stable at high temperatures and “in sustained contact with a wide variety of chemicals, alkalis, dilute acids or oxidizing agents.”⁵⁰ The protocol was modified in ENGS 90 to reduce the percent weight of nylon-6 in the synthesis mixture to decrease the viscosity of the solution, which allows for more consistent pipetting of the polymer into wells (Supplementary Materials 3. Final Protocol – MIP-NAA).

4.2.2 Refining the Approach to Achieve Polymer Uniformity and Repeatability

Polymer composition, volume, and conditions were optimized through experimentation to work toward reliable MIP manufacturing (Supplementary Materials 7. Calculations 2). This iterative experimental workflow was used to determine a manufacturing process that consistently produced uniform, thin, and porous MIPs across the plate wells. Surface morphology and thickness affect the number and accessibility of template recognition sites in the MIP, which need to be highly controlled to fully de-risk the technology. Plates of MIPs with varying compositions were made and assessed on four qualitative criteria: evenness along the bottom of the well, evenness on the side of the wells, gaps along the rim, and congealing along the rim. Each plate was assigned a number from one to three based on success in each category, and these numbers were plugged into alternatives matrices (Appendix A Figures 1 and 2). Scores from the matrices were used to determine the final manufacturing process for the MIP.

4.3 MIP Morphology and Chemistry

4.3.1 SEM

The team used a Tescan Vega 3 Scanning Electron Microscope (SEM) to characterize MIP surface morphology. The literature demonstrates that MIP surface morphology is critical to the efficiency of template adsorption and resorption.^{10,11} The team captured images of bulk nylon-6 MIPs and nonimprinted polymers (NIPs) synthesized in the first round of experimentation (Figure 2; Appendix C Figure 1). The pictures show that the MIPs consist of clustered sphere-like groupings with visible pores between them. In contrast, the NIPs appear to have shear edges where only upon magnification can one visualize small pores in the polymer. This characteristic increased porosity of MIPs versus NIPs is likely due to aggregation monomer-template complexing.¹ Overall, porosity ensures that the MIP will more readily adsorb and desorb analyte in solution, potentially decreasing the MIP-NAA time-to-result.

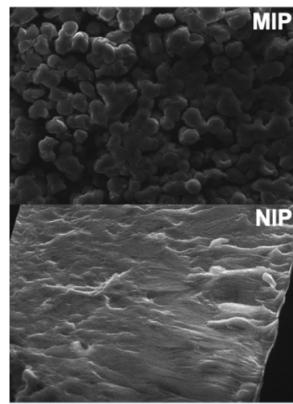


Figure 2 SEM images of nylon-6 MIP and NIP scaled to 100μm.

4.3.2 FTIR

Fourier Transform Infrared Spectroscopy (FTIR) is commonly used to investigate chemical interactions between the template and monomer in MIPs.^{51,52,53} Spectra obtained using a Thermo Scientific iN-10 FTIR microscope in ENGS 89 proved that the MIP captured leucine via characterization of nylon-6 bonding (Appendix B). Shifting in the 3000 cm⁻¹ (C-H and N-H stretching) and 1500 cm⁻¹ (C-H bending) regions are of particular interest due to their indication of the amide-hydrogen bonding that is characteristic of nylon-6 in the presence of amino acids.^{54,55} Experimental results for MIP pre-template-wash, post-template-wash, and post-template-reintroduction closely match nylon-6 imprinted polymer spectra in the literature (Figure 3; Appendix B Figure 1). The shifting from MIP pre-wash to post-wash, an approximately 10% drop in transmission, indicates that the template molecule was at least partially removed during the acetic acid solution wash. The team theorizes that this drop in transmission correlates with

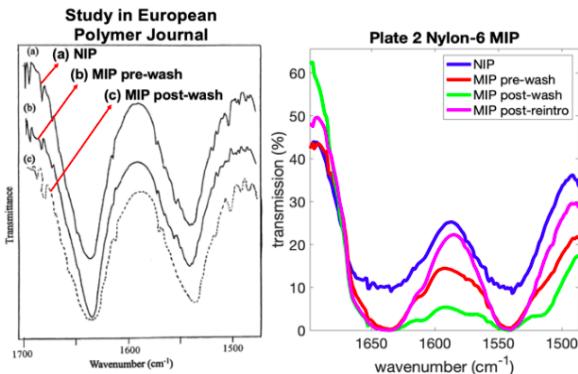


Figure 3 (Left) Reddy et al. FTIR spectra of nylon-6 MIP and NIP. (Right) FTIR spectra of synthesized nylon-6 MIP and NIP.

project feasibility but did not accurately quantify template extraction and adsorption. The team also used FTIR to characterize the nylon-6 MIPs before and after soaking in sample broths for 5 to 60 minutes (Supplementary Materials 4. Lab Report 7). The spectra show that there were not any significant changes in the molecular structure or bonding of the polymer after sitting in slightly acidic solutions for up to one hour (Appendix D).

4.4 Gold Nanoparticle (AuNP) Transduction

4.4.1 Proposed Mechanism

The team developed a multi-step reduction process to synthesize AuNPs for the colorimetric quantification of amino acids. Numerous literature sources demonstrate nanoparticle agglomeration caused by different amino acids.^{56,57,58,59,60,61,62,63} Many of these sources take advantage of complex ligand conjugation of AuNPs, which complicate experiments and increase costs. Alternatively, Berghian-Grosan et al. use an amino acid as the reducing agent for nanoparticle formation without the addition of a conventional reducing agent, such as sodium citrate or sodium borohydride.⁶⁴ They found that employing the amino acid as sole reducing agent caused significant particle agglomeration, leading to a redshift in the absorbance. They observed less agglomeration when the amino acid was used as a secondary reducing agent and capping ligand in the presence of a primary reducing agent, sodium citrate. Therefore, the team developed a nanoparticle synthesis protocol in which the reducing agent, sodium borohydride, is limited, allowing for continued reduction of the gold (III) chloride upon the addition of leucine (Supplementary Materials 4. Lab Report 5). The team proposes that absorbance redshift is caused by two mechanisms: 1) nanoparticle growth due to the reduction of gold III ions in solution and 2) leucine's hydrophobic side chain acting as a ligand that pulls the nanoparticles together. This effect was demonstrated across batches of nanoparticles to be linearly correlated with amino acid concentration which will be discussed in further detail in *Section 4.4.3*.

4.4.2 Preliminary Experiment with Prepared Standards

Based on the literature and experimentation, the team was able to create nanoparticles that would agglomerate in the presence of the leucine template molecule within the desired concentration range (Supplementary Materials 4. Lab Report 5).^{65,66} For consistency between plots, the analyte concentrations on the x-axis for each standard curve represent the concentration of leucine in solution after the standards were added to nanoparticle solution. Upon the addition of higher concentrations of amino acid, an observable redshift in the absorbance spectrum was realized (Figure 4 (Left)). This redshift was quantified by taking the ratio of the absorbance at 570nm to that at 460 nm for reasons explained in *Section 4.5*. This transduction effect showed excellent

stronger bonding between nylon-6 molecules once the template is removed. The MIP post-reintroduction spectrum shows an approximately 20% increase in transmission compared to the MIP post-template-wash, suggesting that MIP recognition of the amino acid upon reintroduction was successful. It should be noted that the FTIR is sensitive to sample thickness and opacity. Experiments aimed at quantifying the relationship between polymer thickness and nylon-6-characteristic FTIR peak heights were inconclusive (Supplementary Materials 4. Lab Report 1). Preliminary FTIR results proved

linearity with R^2 values greater than 0.95 at in-well leucine concentrations across two to three orders of magnitude in difference (Figure 4 (Right)).

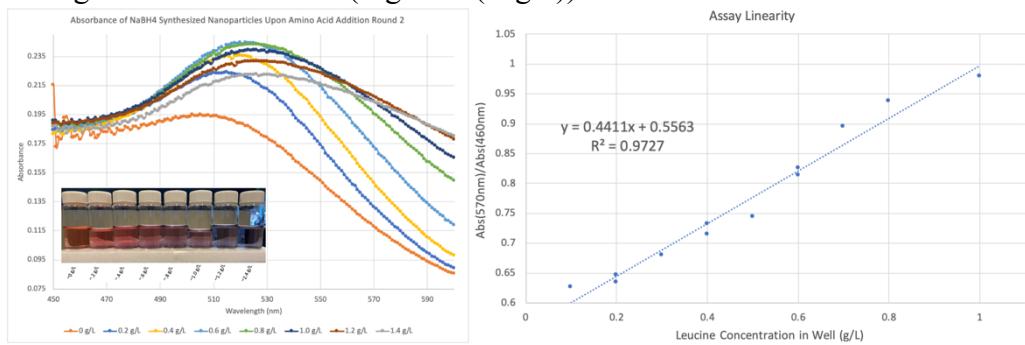


Figure 4 (Left) Colorimetric redshifting of AuNPs due to agglomeration at higher concentrations of leucine in solution. (Right) Signal processing of absorbance data to create linear response vs. concentration curve.

4.4.3 Repeatability

Throughout experimentation, the team synthesized several batches of AuNPs and created their corresponding standard curves (Section 4.4.2; Appendix E Table 1). Linearity ($R^2 > 0.97$) was consistent across small variations in AuNP solution composition, but parameters of the linear fit (i.e. slope, y-intercept) varied. As such, the team recommends that fresh standard curves be created for each test. Additionally, the AuNPs were tested across various in-well concentrations to assess the full linear range of the batches. Given that the standards were diluted to a ratio of 15 μ L standards to 200 μ L AuNPs, the full realized linear range of undiluted sample is 0.19 g/L (0.08 g/L diluted) to 15.05 g/L (1.05 g/L diluted). It should be noted that if the sodium borohydride concentration greatly exceeds the designated value of 0.0011 g from the final protocol, the AuNPs show minimal response within the designated leucine concentration range. It is hypothesized that excess sodium borohydride causes the gold (III) chloride to be fully reduced, which inhibits the reduction effects from the addition of leucine (Section 4.4.1). The team also qualitatively observed that the AuNP synthesis protocol is highly sensitive to slight errors, such as failing to thoroughly wash all lab equipment (i.e. flask, scoopula, etc.).

4.4.4 Longevity and pH Effects

The team studied nanoparticle efficacy over time because every batch of nanoparticles produced can cover the processing of approximately 500 samples. Nanoparticles were found to be effective from one hour to five days post synthesis (Appendix E Figure 1). Notably, the slope of the response vs. concentration curve decreases with time, which confirms that a new standard curve should be made for each new analysis. Qualitatively, it was noted that high amounts of light exposure quickly decreased nanoparticle effectiveness, which is typical of gold nanoparticle degradation. Therefore, any vials containing nanoparticles were wrapped in tin foil and stored in the refrigerator. Additionally, the team wanted to verify that the redshift response from increasing amino acid concentration was attributed to an interaction with the analyte, not to a decrease in pH. The grounds for this investigation were based on several papers that compare colorimetric response of AuNPs to changes in pH.^{67,68,69} Standard solutions were made at pH 7 to compare to non-pH-controlled solutions. As shown in Appendix E Figure 2, the standard curves and deviations for pH controlled and not controlled solutions show great similarity. Therefore, the assay was not significantly affected by variation in pH ranging from 6 to 7.

4.4.5 Effects of Interfering Molecules

The team tested the robustness of the NAA to the most prominent interfering molecules in the sponsor's fermentation broth, including glucose, ammonium, carboxylic acids, and side-product amino acids. Research indicates that glucose, ammonium, and carboxylic acid levels could vary in fermentation broth from 5 to 260 g/L, 0.5 to 2.0 g/L, and 5 to 70 g/L respectively.^{70,71,72,73,74} Therefore, solutions of the interfering molecules were added to the wells to emulate broth concentrations (Figure 5). The presence of glucose up to 21 g/L had little effect on the linear curve with R^2 values above 0.95. Similar robustness in glucose was demonstrated by Shen et al. with their tetracycline assay based on gold nanoparticle-induced growth.⁷⁵ Although the addition of ammonium chloride lead to a decrease in the slope and y-intercept of the standard curves, it still showed remarkable linearity with R^2 values above 0.96. However, Aryal et al. realized the opposite effect on nanoparticle agglomeration and the accompanying redshift with the addition of NaCl, another electrolyte.⁷⁶ Niidome et al., in contrast, found that treatment of gold nanorods with various ammonium salts inhibited particle growth and led to a decrease in peak wavelength.⁷⁷ Ultimately, the chemical mechanism of ammonium interactions should be reviewed further. Lastly, acetic acid greatly influenced the nanoparticle absorbance properties even at low concentrations. This is expected given the proposed mechanism of agglomeration involving electronegative oxygen of the amino group reducing the gold III chloride (*Section 4.4.1*). Carboxylic acids are likely also capable of causing this phenomenon as demonstrated by the higher than expected absorbance ratios shown in Figure 5 (Right). For more information, see Supplementary Materials 4. Lab Report 9.

4.5 Signal Analysis

4.5.1 Choosing a Metric

The shift in peak absorbance of AuNPs due to a change in particle size or agglomeration is well characterized and can be controlled by the addition of particular analytes, making AuNPs promising tools for transduction in biochemical sensors.^{78,79,80,81} However, the metric used to compare changes in absorption spectra of AuNPs varies with the analyte of interest and has not been established for amino acids. Two colorimetric assays for small organic molecules (1-hydroxypyrene and dopamine) use the shift in peak AuNP absorbance as transduction mechanisms in their sensors.^{82,83} Other studies use ratios of absorbances, commonly A_{620}/A_{520} , to distinguish changes in the AuNP absorption spectra from changes in analyte concentration.⁸⁴ The team investigated three metrics to potentially linearly correlate nanoparticle agglomeration to the concentration of leucine: 1) the peak absorbance, 2) the ratio of the absorbance A_{570}/A_{510} , and 3) the ratio of the absorbance A_{570}/A_{460} . In choosing these ratios, the team sought to home in on the

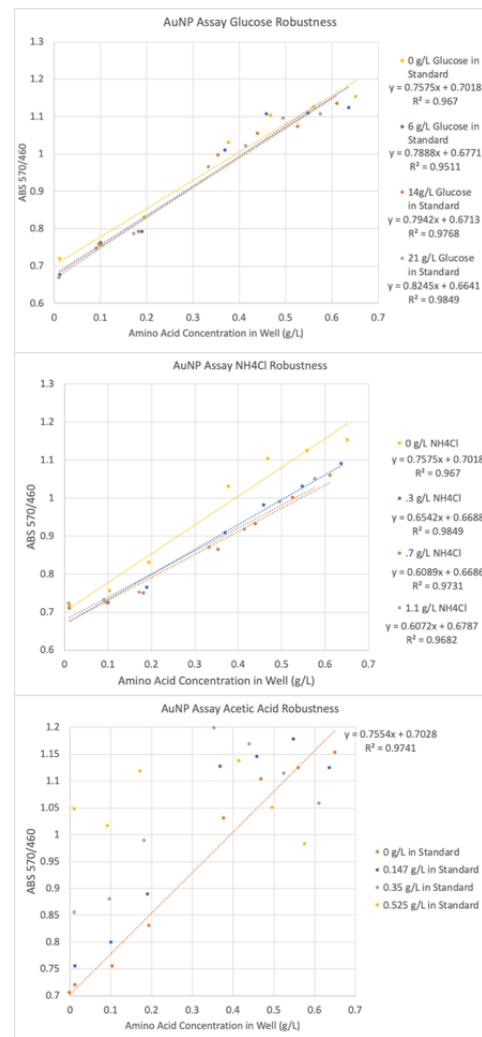


Figure 5 Agglomeration Assay Interference Testing. (Top) Glucose-spiked standards. (Middle) NH₄Cl-spiked standards. (Bottom) Acetic Acid-spiked standards.

variability in absorption caused by changes in analyte concentration (A_{570}) and “normalize” to variability not due to analyte concentration (A_{460} and A_{510}).

To compare the three metrics, standard solutions of leucine and water were prepared (0 to 14 g/L which were then diluted to 0 to 1.4 g/L) and combined with freshly synthesized AuNPs. Absorbance spectra were measured for each well, the three metrics calculated, and standard curves (metric v. concentration leucine) created using a custom MATLAB script (Supplementary Materials 4. Lab Report 6). This procedure was repeated using standards that were pH controlled by adding a small amount of sodium hydroxide (pH ~ 7 compared to $6 < \text{pH} < 7$ of the non-pH-controlled standards). Metrics were evaluated by the linearity of their standard curve (R^2) and the change in linear fit for pH-controlled versus non-pH-controlled standards. The ratio A_{570}/A_{460} performed best, resulting in a high R^2 of 0.976 for both types of standards and only a very small shift in slope (+9%) between pH-controlled and non-controlled. For these reasons, the ratio A_{570}/A_{460} was chosen as the metric moving forward with the MIP-NAA.

4.5.2 Statistics for Assay Development

A set of standard leucine and water solutions (0.1 to 5 g/L) were prepared to test the assay and conduct statistical analyses. Plates were prepared following the configuration in Appendix F Figure 1A (N=12), where standards were pipetted into the wells first (20 $\mu\text{L}/\text{well}$), followed by the AuNP solution (180 $\mu\text{L}/\text{well}$), which diluted the amino acid by a factor of 10 to 1.

The overall workflow of statistical analysis consisted of 1) Calculating the ratio A_{570}/A_{460} for each well, 2) Computing descriptive statistics across replicates (i.e. average and standard deviation for N=12), 3) Checking that the data can be approximated as coming from a normally distributed population (Anderson-Darling test, probability plots), 4) Creating the ratio versus concentration curve and a linear curve fit, and 5) Conducting a power analysis to determine (A) the number of samples required to distinguish between sample concentrations at constant 0.25 g/L intervals and (B) between sample concentrations and sample concentrations plus 10% (i.e. between 0.1 and 0.11 g/L for the lowest concentration) (Appendix F Figure 1). The linear curve fit equation was used to determine the ratio precision needed to attain the particular unit of precision in concentration. 0.25 g/L was chosen as an appropriate constant unit of precision across concentrations because it is 10% of the middle concentration value (2.5 g/L), whereas 10% precision fits the project’s goal specification of achieving concentrations within $\pm 10\%$.

The power test showed that it is very difficult to attain 10% precision for the lowest end of the range specified by the sponsor, requiring a sample size over 4000 (Appendix F Table 1). However, for the middle-to-high end of the concentration range, it is more feasible, requiring 6 to 24 replicates. For a constant precision of ± 0.25 g/L, 24 replicates would ensure a power of 0.80 across the concentration range, but that unit of precision is unreasonable for concentrations below 1 g/L. In their experimental iterations, the group primarily tested with 3 to 12 replicates, but after conducting this analysis, the team recommends that further development of the assay be focused on samples of concentration greater than 3 g/L, where 10% precision and a power of 0.80 can be attained with as little as 9 replicates. Finally, it should be noted that this analysis was limited to testing with leucine standards in water (i.e. no fermentation broth or MIP analyte capture). Further development of the MIP-NAA would require a similar statistical analysis to be completed for the results from the full workflow.

4.5.3 Proposed Workflow for Final Signal Analysis Protocol

The final deliverable to the sponsor includes a comprehensive protocol for how to use the MIP-NAA to estimate the concentration of an unknown sample (Supplementary Materials 3. Final

Protocol - MIP-NAA). It consists broadly of MIP synthesis, amino acid standards preparation, AuNP solution preparation, analyte sensing, and signal analysis. This section describes analyte sensing and signal analysis in further detail.

First, 20 μL of standard samples and unknown samples are pipetted into the plate in the format illustrated in Figure 6A. 200 μL of AuNP solution is added to each of the wells, and the plate is allowed to sit for 30 minutes. After the 30-minute wait time, the plate reader is used to measure the absorbance at 570 nm and 460 nm for each well. That data is imported into MATLAB and analyzed using a custom script (Supplementary Materials 5. Code). The script ignores empty wells, calculates A_{570}/A_{460} for each well, removes outliers for each sample (i.e. samples with $A_{570}/A_{460} > 3\sigma$ away from the mean across replicates), computes the average and standard

deviation for each sample, establishes a standard curve, and estimates the concentrations of unknowns based on the standard curve. The step to remove outliers assumes the data for each concentration has an approximately normal underlying population distribution, as was validated using the Anderson-Darling test (Supplementary Materials 4. Lab Report 8). A limitation of using $N=3$ is that the distribution computed for a set of replicates could be easily skewed toward an outlier, making it difficult to remove. As such, only

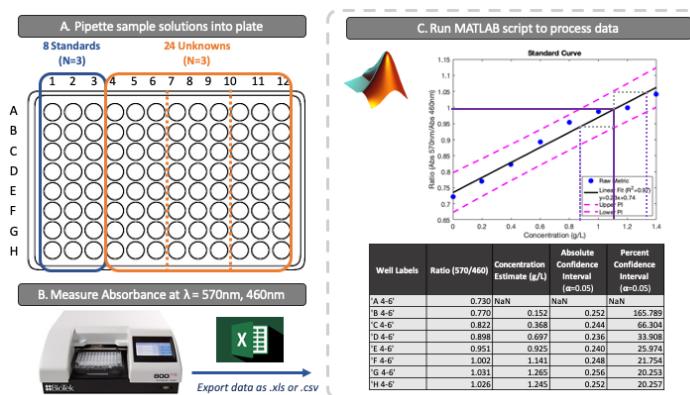


Figure 6 Summary of sensing workflow; A) Plate template for standards and unknowns, B) Transduction to measured change in sample absorbance, C) Outputs of signal processing using MATLAB Note: Concentrations that fall outside of the range of standards are left as NaN ("Not a Number").

severe outliers are removed for this sample size. Replacing single-channel pipettes with multi-channel pipettes, or converting to an automated pipetting system, would allow for more replicates to be tested and make outlier removal more effective.

The concentration of unknown samples is computed using the linear fit ascribed to the plot of standard sample A_{570}/A_{460} versus concentration. The estimate is given "95% confidence intervals" using the 95% prediction intervals of the standard curve linear fit as illustrated in Figure 6C. The final output of the code consists of the standard curve and a table summarizing the ratio A_{570}/A_{460} , concentration estimate, and confidence intervals (absolute and percent based) for each unknown sample.

5. Integrated Assay Results

5.1 Initial Results for Full Workflow (Leucine in Water Samples)

Initial tests of the fully described MIP-NAA system were conducted with leucine and water standards (Supplementary Materials 4. Lab Report 10). To show proof-of-concept, both the adsorption and extraction phases were allowed 24 hours to ensure minimal variation in diffusion through the MIP. The largest source of variability in these tests was the ability to remove all of the template from the MIP and the residual liquid from the wells between the adsorption and extraction phases. Template-removal protocol 1 consisted of soaking the polymer in water for 90 minutes. Template-removal protocol 2 included a 24-hour soak. Residual liquid was removed in

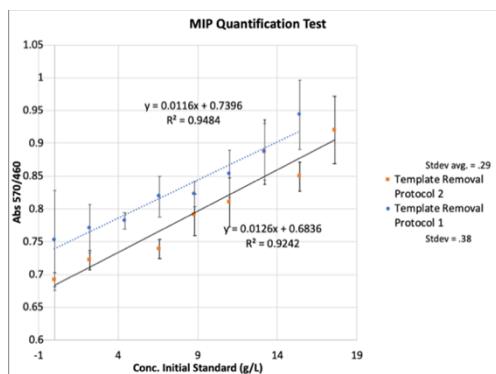


Figure 7 Results of full MIP-NAA protocol with water standards. Smaller error bars in protocol 2 indicate more effective liquid removal before extraction phase.

protocol 1 by dumping out the leucine standards from the well and air drying. In protocol 2, residual liquid was dried using sprayed air aimed at the upside-down plate to allow residual liquid to fall out of the wells. This led to an average standard deviation decrease from 0.038 to 0.029 for A_{570}/A_{460} across the concentration range (Figure 7). This reduction in standard deviation decreases the concentration quantification error from $\pm 18.6\text{-}49.6\%$ to $\pm 13.1\text{-}34.9\%$ for 17.6 to 6.6 g/L leucine in water. While this is not quite within the $\pm 10\%$ accuracy threshold, it shows that slight protocol optimization significantly decreases error. Overall, the team found that the only way to effectively remove the residual liquid was to use an intermediate liquid rinse, which is discussed in *Section 5.2*. The full MIP-NAA workflow requires repeated adsorption and extraction of the template molecule in and out of the MIP, introducing considerable variability to the NAA results. As such, the team sought to quantify the effectiveness of template washing post-synthesis and template reintroduction.

Three attempts were made to quantify amino acids in solution to analyze MIP adsorption/resorption effectiveness: 1) the team attempted to develop a method for quantifying FTIR results (Supplementary Materials 4. Lab Report 1), 2) the team purchased an amino acid quantification assay (AAA Kit) from CellBiolabs (Supplementary Materials 4. Lab Report 3), and 3) the team used their developed NAA protocol (Appendix G). Only method 3 worked well. Results suggest that template-washing including an overnight soak in water is effective at removing most of the analyte. Analysis of the reintroduction solution led to two conclusions: in aqueous solution, most of the leucine is likely adsorbed into the MIP within a few hours, and adding a hydrophobic film to the MIP surface to prevent other broth components from adhering severely hampers the rate and potentially the extent of leucine adsorption (Appendix G).

5.2 Initial Results for Full Workflow (Leucine in Fermentation Broth Samples)

The team set out to test the MIP-NAA protocol on yeast peptone dextrose (YPD) broth samples obtained from Professor Jiwon Lee's laboratory and DMC's bacterial broth spiked with leucine (Supplementary Materials 4. Lab Report 11). Ultimately, the various interfering molecules in these broth samples necessitated the optimization of several factors for use: adsorption and extraction time, solvent washes to remove the interfering molecules, and the addition of a hydrophobic coating. As discussed in *Section 5.1*, the addition of the trichlorosilane hydrophobic coating used to prevent proteins in solution from sticking to the nylon-6 surface also negatively impacted leucine diffusion into the MIP. For YPD broth, response v. concentration shifting was achieved with 2 μL of trichlorosilane in each well, allowing for 90 minutes of adsorption and extraction, and rinsing with water (Figure 8 (Left)).

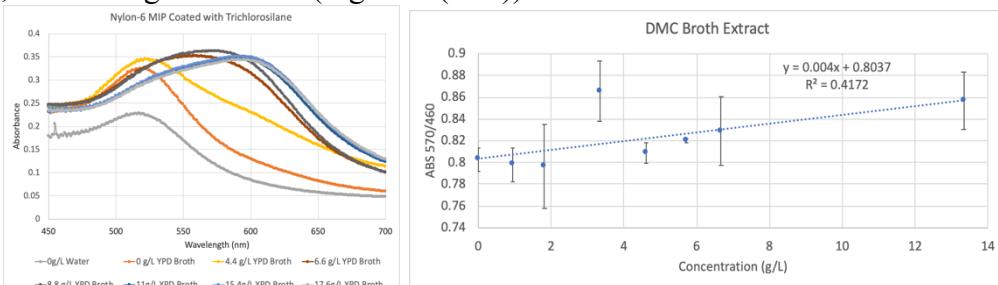


Figure 8 (Left) Preliminary proof-of-concept of MIP-NAA system in YPD broth. (Right) Trial of protocol in DMC ultrafiltrate with alanine.

The same protocol was tried on DMC seed medium and ultrafiltrate. Overall, the adsorption at 90 minutes of DMC broth proved to be quite low (Figure 8 (Right)). To combat this, the team experimented without the hydrophobic coating and instead rinsing with methanol between adsorption and extraction to remove interfering molecules from the MIP surface without removing the adsorbed leucine. Most organic acids are quite soluble in methanol, while nonpolar aliphatic amino acids have a low solubility of 0.012 to 0.013 g/L compared to leucine's solubility in water at 24.26 g/L.^{85,86} Appendix H Table 1 shows a recovery range of 5 to 30% of the initial concentration with the described protocol after 45 minutes. If given more time to work with the broth, a more comprehensive optimization of the adsorption, rinsing, and extraction protocol specific to DMC's broth would have been conducted (Supplementary Materials 8. Recommended Experiments). For instance, Xiaolan et al. developed a nylon-6 MIP stir bar for monocrotophos recovery from in-soil samples and characterized their system with dichloromethane, acetonitrile, methanol, and water to attain an optimized protocol.⁸⁷

5.3 Assay Specificity and Adaptability

The team completed iterative testing to validate the ability of the MIP-NAA to achieve linear standard curves using leucine in water. Next, the team prepared isoleucine standards in water to investigate the adaptability of the assay to other amino acids. The team found that the standard curve for isoleucine appeared linear at very low concentrations, but shortly saturated, much faster than the leucine standard curves (Appendix I Figure 1). As such, the team believes that it is possible to adapt the assay to other amino acids, but the dilution factor of the sample will likely need to be optimized to achieve linear standard curves.

Next, the team sought to confirm the specificity of their prepared MIPs between the structural isomers, isoleucine and leucine. This experiment also tested the transferability of the team's MIP synthesis and analyte capture workflow beyond leucine as the template molecule. Two plates of MIPs were synthesized, one using leucine as a template molecule and the other using isoleucine. Moderately concentrated solutions of leucine in water and isoleucine in water (4.3 g/L, 4.5 g/L respectively) were prepared, and 100 μ L of each were pipetted into a row on the leucine MIP plate and the isoleucine MIP plate ($N=12$) and left overnight. Samples were taken from each well and combined with the AuNP solution to measure and calculate A_{570}/A_{460} . These samples and corresponding metrics were compared with samples of the same starting concentrations to determine whether the change in the metric A_{570}/A_{460} was statistically significant (Appendix I Table 2). Statistical significance (reject or fail to reject the null hypothesis) of this change in A_{570}/A_{460} was used as a proxy for determining whether the MIPs adsorbed considerable amounts of the analyte in the standard solution. The results were conclusive in showing that the leucine plate specifically adsorbed leucine, but little to no isoleucine. Unfortunately, the team was unable to obtain conclusive results to support the claim that the isoleucine plate adsorbs significant isoleucine, and only weak evidence to support the claim that it does not adsorb significant leucine. The team hypothesizes that the workflow for isoleucine MIP synthesis may need modification from that of the leucine synthesis. However, the literature cites extensive evidence supporting MIP specificity and adaptability between template-molecules.^{88,89} The team believes that if DMC were to repeat the experiment, minding sufficient template washing following synthesis, they could validate the specificity of the isoleucine MIP.

5.4 Review of Assay Results Against Specifications

In order of importance, the project specifications are: cost, accuracy, repeatability, dynamic range, specificity, robustness, adaptability, and time-to-result (Appendix J Figure 1).

The breakdown of per-plate costs is: \$5.05 for MIP synthesis materials, \$0.61 for AuNP agglomeration solution, \$0.02 for standard solutions, and \$2.56 for washing and resorption materials. In total, this equates to 9¢ per sample per well (Supplementary Materials 1. Head-to-Head Breakdown). Power tests showed that attaining 10% precision for the middle-to-high end of the concentration was feasible with 6 to 24 replicates, while attaining the same precision level was calculated to be more difficult for the lowest end of the range (*Section 4.5.2* and Appendix F). Preliminary experimental results show significant promise for achieving repeatability and specificity, consistency between wells, plates, and analytes, and the ability to achieve a signal corresponding with the analyte of interest that is 10x greater than those of any interfering molecules (*Section 4.4.3*; *Section 5.3*). The NAA demonstrated robustness in the presence of glucose and ammonium, which are possible interfering molecules, but was ineffective with carboxylic acids (*Section 4.4.5*). Finally, the protocol allows for the production and analysis of 1000 samples in under a week (Supplementary Materials 1. Head-to-Head Breakdown).

The team ordered an amino acid assay kit (AAA Kit) to quantify free L-amino acids in solution to compare to the proposed MIP solution. Such colorimetric assays have been used as a cost-effective and high-throughput mechanism of molecular quantification. Many rely on employing enzymatic reactions to elicit the production of a colorimetric probe. The AAA kit uses L-amino acid oxidase to deaminate alanine, the amino acid template of interest. It then detects the released hydrogen peroxide through a horseradish peroxidase catalyzed reaction with a probe molecule. During preliminary experimentation, sets of known leucine and alanine standard solutions were prepared. Although the alanine standard worked well ($R^2 > 0.99$), the leucine standard yielded non-linear data (Supplementary Materials 4. Lab Report 3). After several iterations of leucine standard quantification using the AAA kit, the team concluded that the assay was highly sensitive to minute changes in pH ($6 < \text{pH} < 7$), that the protocol was ineffective for leucine as written (assay incubation time needed to be reduced from 90 to 5 minutes), and that kit results were inconsistent between tests. For less than \$10 per plate, the MIP-NAA outperformed the AAA kit, which costs \$400, across every metric tested.

6. Sustainability and Safety Analysis

The synthesis of MIPs requires the use of strong acids and other potentially harmful reagents. As such, the team consistently reviewed safety data sheets (SDS) of all reagents prior to use.

Appendix K Table 1 lists the expected quantities of reagents required by the MIP-NAA over the course of a year. It should be noted that the protocol calls for corrosive and combustible chemicals. Therefore, those following the protocol should wear the proper PPE, be properly trained, and be familiar with the risks of exposure and the methods of disposal. Nylon-6 is non-hazardous and non-corrosive.⁹⁰ It is non-biodegradable, and the industry standard is to recycle it.⁹¹ The amino acid is non-hazardous and non-corrosive. Proper disposal of small quantities involves discarding in the trash.⁹² Formic and acetic acids are hazardous and combustible. Waste should be contained and disposed of at the proper processing plant. Spills should be neutralized with soda-ash or soda-lime.^{93,94} Both acids must be handled in a fume hood equipped with a condenser, trap, or scrubber to collect and contain the waste solvent and toxic vapors.⁹⁵ Gold chloride solution is hazardous and non-combustible.⁹⁶ Surplus solutions should be sent to a licensed disposal company. Sodium borohydride is hazardous and combustible.⁹⁷ It should be quenched with isopropanol and disposed of as hazardous waste. Trichlorosilane is hazardous and combustible.⁹⁸ Waste should be contained and disposed of at the proper waste processing plant. Although the assay protocol calls for chemicals that may be harmful to human and/or

environmental health, the team does not anticipate any severe health and environmental safety consequences with proper handling and waste disposal.

7. Intellectual Property (IP) Plan

The team made an effort throughout the project to ensure that the developed MIP-based assay sensor solution does not infringe on any existing patents. Specifically, there is a website called MIPdatabase.com that catalogues most MIP patents. After examining this source and others, such as the United States Patent Office, the team has no current knowledge of an existing patent covering 96-well MIP-based plasmonic assays for biofermentation product quantification. Furthermore, the coupling of MIP specificity with nanoparticle agglomeration in the presence of the analyte of interest is a seemingly novel technology that has yet to be patented.

8. Pro Forma Financials and Implementation Plan

The MIP-NAA can be used by DMC first, as an internal analysis tool to effectively expedite and lower the costs of product R&D and later, as a potentially stand-alone commercial product. The team's pro forma analysis along with a full justification of the assumptions made is found in Supplementary Materials 6. Full MIP-NAA Pro Forma. This analysis estimates that the integration of the proposed analytical method would provide a net present value (NPV) of around one million dollars over the next ten years, or 10.6% of the company's Series A Financing received this past summer. This value is derived from four sources: increased revenues from early product to market times, increased revenues from selling the assays themselves, reduced fixed capital expenses, and reduced variable R&D expenses.

The team recognizes several risks remain that impact the full net present value of the proposed MIP-NAA. Firstly, the MIP adsorptive extraction should be optimized for DMC's fermentation broth given its unique profile of organic acids. The team iterated to produce a MIP synthesis protocol that led to better uniformity among MIPs across wells, and they identified specific components in the fermentation broth that stick to the polymer surface, causing interference during signal analysis of the analyte. The team's research suggests that acidic pH modulation and more viscous stirring might lead to more effective amino acid adsorption. Additionally, solvent rinses between adsorption and extraction using methanol, ethanol, and/or acetonitrile could effectively lead to better removal of interfering molecules without loss of the analyte of interest. Lastly, testing the MIP-NAA functionality with amino acids other than leucine and isoleucine, as well as other potential fermentation products, would help to demonstrate its scalability across product pipelines (Supplementary Materials 8. Recommended Experiments).

Further, the incorporation of the isolated NAA into the chromatography downstream workflow at DMC has the potential to provide immediate value to the company. Their current analysis of the fragments in chromatography separations requires either a total dissolved solids analysis or UPLC. DMC has attempted to replace such time- and resource-intensive methods with enzymatic assays with little success due to their unreliability (*Section 5.4*). As such, the team proposes testing the NAA for quantification post-chromatography separation where amino acid samples are relatively pure. The NAA has several benefits regarding experimental cost, sample usage, hands-on time, and experimental feedback time compared to total dissolved solids analysis. Firstly, 100 weight boats found online at Cole-Parmer cost \$20, whereas usage of the NAA costs around \$5.70 for 100 samples, allowing for some modest cost savings. Additionally, the amount of sample from each fragment taken off the column would be in the 10 to 30 μL range compared to the 3 to 7 mL range used for total dissolved solids analysis. This makes the calculation of overall collection yields easier in the process optimization phase. Lastly, total

dissolved solids analysis involves: 1) labeling and weighing each weight boat, 2) transferring a specific volume of liquid, 3) drying the boats overnight in the vacuum oven, and 4) re-weighing all of the boats. Instead, using the NAA with a multichannel pipette, one could yield results nearly instantaneously, and the workflow from nanoparticle synthesis to reading the absorbance measurements would take at most 25 minutes of hands-on time. After running a standard curve, one could process as many chromatography fragments as needed. Future steps towards bettering workflow incorporation and validation of the NAA for this purpose would include head-to-head analysis against with the UPLC, investigating potential stabilizing additives or storage procedures for the AuNP solution, and finally, determining the optimal dilution factor for different amino acid analytes. The team's work confirms the utility of the NAA for leucine and isoleucine, and several studies suggest that the NAA should function across numerous other amino acids such as lysine, cysteine, asparagine, and tryptophan.^{99,100, 101,102}

9. Future Recommendations

The team made significant strides developing the MIP-NAA, incorporating nylon-6 MIPs for specific amino acid capture and AuNPs for transduction of capture into a measurable, linear signal. Overall, the team believes that the MIP-NAA exhibits distinct advantages over SOA assays, including a much lower cost per plate and greater robustness against small changes in pH. The technology will be delivered for scale-up and implementation at DMC in the form of this report outlining the development of the MIP-NAA and the progress made toward accomplishing the specifications provided by the sponsor, documentation of polymer and AuNP synthesis protocols, and MATLAB code for spectrometer data analysis. The team outlined recommended future experiments to assist the technology transfer (Supplementary Materials 8. Recommended Experiments). The most critical experiments include determining the rate of template extraction and adsorption, establishing an effective washing protocol to remove interfering molecules, and identifying the number of replicates needed to achieve the desired accuracy and precision. The team believes that the results from these experiments will help determine DMC's path forward regarding full MIP-NAA implementation.

The team recommends that DMC move forward with 1) developing the assay as a screening tool for their fermentation broth samples, resulting in the need for less frequent measurements on the costly and time-intensive UPLC, and/or 2) separating the AuNP transduction mechanism from the MIP and directly integrating it into their chromatography workflow. Option 2 would require a low time and resource investment for DMC that could, in turn, provide them significant value over the current method for analyte quantification following purification. The team believes that DMC is well positioned to continue development of the MIP-NAA and/or the NAA given the technical backgrounds of their employees and their established laboratory facilities (i.e. multi-channel pipettes instead of single-channel). However, the time and cost of developing this novel technology must be weighed against the company's broader strategies and commitments. The team is excited to offer DMC an option that could be implemented straight away and an option that would take a little more effort to develop but has the potential to provide real value.

Acknowledgements

The team would like to thank the following people for their contribution to this project: Jiwon Lee for his ongoing support as advisor, Mary Kay Brown for providing the laboratory facilities, and Barbara Currier for her FTIR expertise. Special thanks to Joseph BelBruno, Amogha Tadimety, and Chuanlong Wang and Fiona Li's lab for their assistance in various troubleshooting aspects of the project.

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Appendix A

Polymer Uniformity & Repeatability Matrices

	Even well-bottom surface (x4)	Even well-sides coating (x3)	No gapping at the rim (x2)	No congealing on the rim (x2)	Total
Plate shaker	3	3	1	2	27
Sonicator	2	1	3	1	19
Static	1	0	3	0	10

Figure 1: Repeatability challenges polymerization matrix.

	Even well-bottom surface (x4)	Even well-sides coating (x3)	No gapping at the rim (x2)	No congealing on the rim (x2)	Captures within range of Agglomeration Assay (x5)	Total
10 µL	1	2	0	3	Further testing needed	16
15 µL	0	0	0	3	Further testing needed	6
20 µL	0	2	0	3	Further testing needed	12
30 µL	1.5	1	1	1	Further testing needed	13
40 µL	3	2	2	2	Further testing needed	26
45 µL	2	2	1	1	Further testing needed	18
60 µL	2	1	3	0	Further testing needed	17
100 µL	2	1	1	3	Further testing needed	19
200 µL	3	3	3	2	Further testing needed	31

Figure 2: Repeatability challenges volume matrix.

Appendix B

Nylon-6 FTIR Results

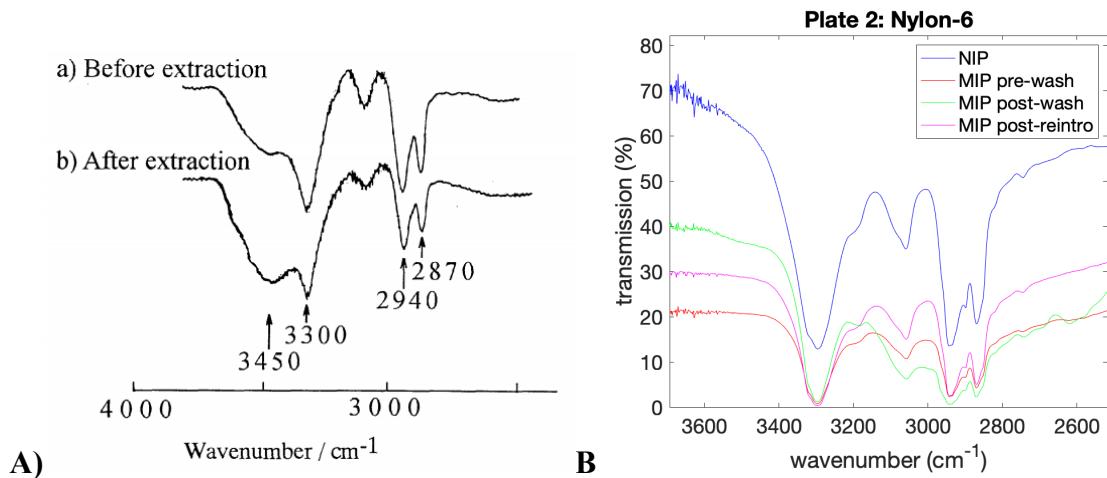


Figure 1: **A)** Literature FTIR Spectral Analysis of L-glutamine-imprinted Nylon-6 (3000 cm^{-1} region): Top curve shows MIP before template extraction, and bottom curve shows MIP after template extraction.¹ **B)** Experimental FTIR Spectral Analysis of Nylon-6 Imprinted Polymer from 20 mL vial for an amino acid close in structure to L-glutamine (3000 cm^{-1} region).

Appendix C

SEM/EDS Polymer Characterization

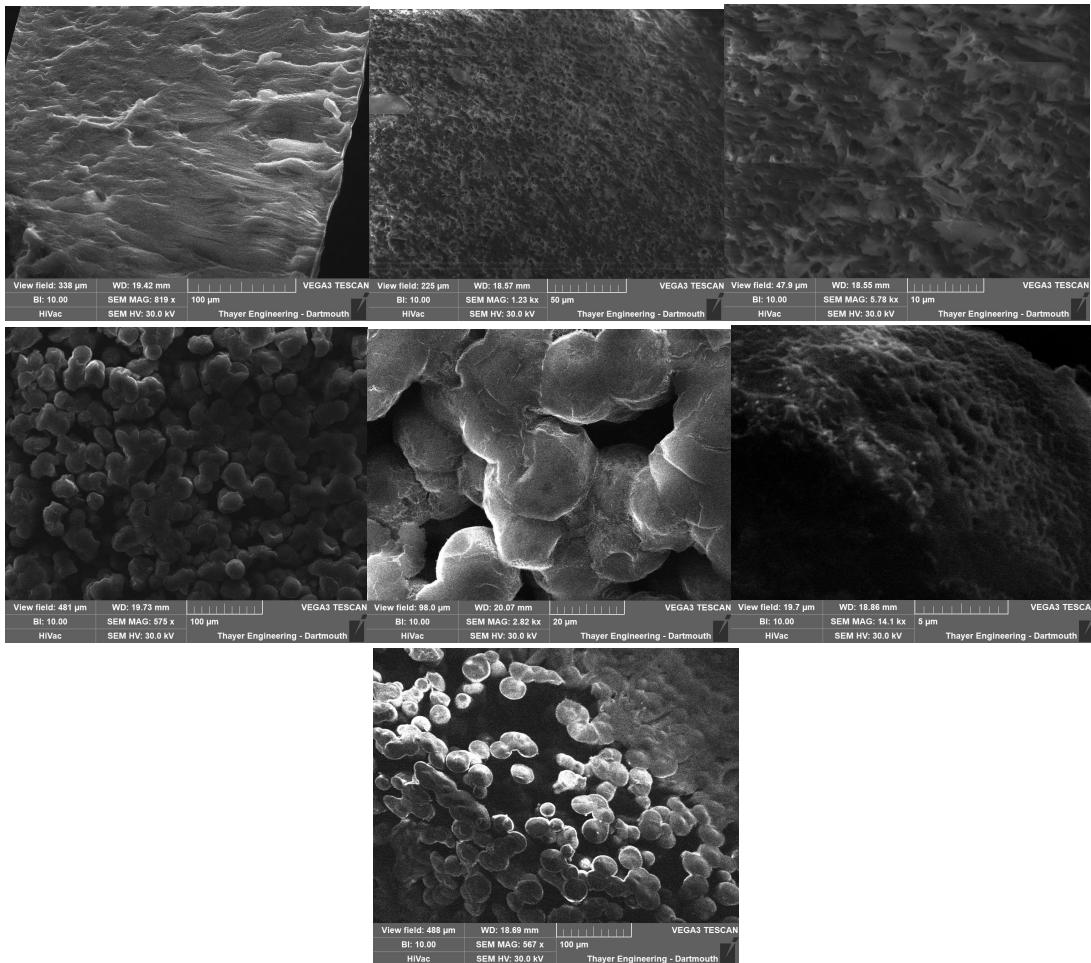


Figure 1: SEM Images of synthesized Nylon-6 MIP vs. NIP. (Top) NIP magnified at 100 μm, 50 μm, and 10 μm scale left, middle, and right respectively. (Middle) MIP magnified at 100 μm, 20 μm, and 5 μm scale left, middle, and right respectively. (Bottom) View of compressed MIP at bottom edge of the well.

Appendix D

FTIR Spectra from Durability Testing

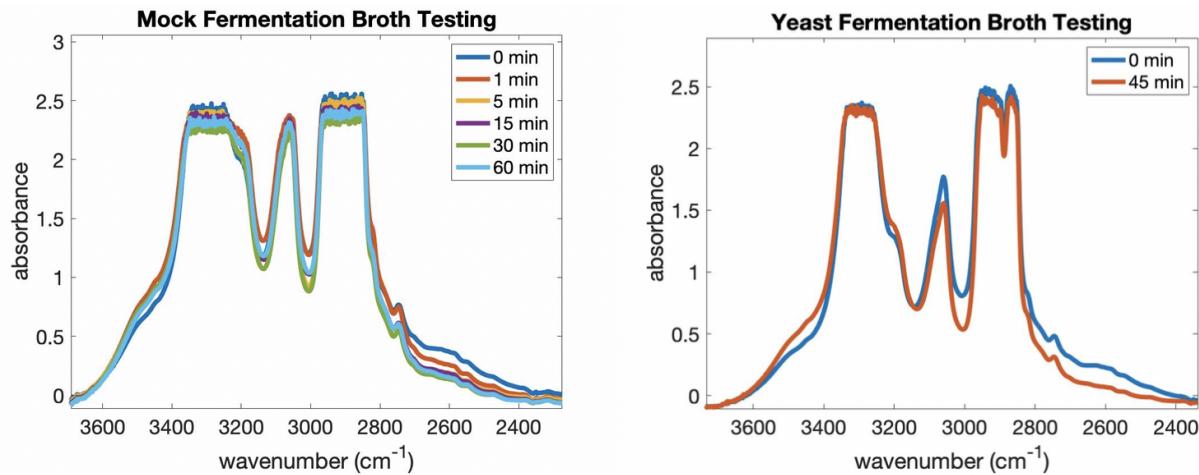


Figure 1: FTIR spectra from polymer durability testing in mock (left) and yeast (right) fermentation broths.

Appendix E

Nanoparticle Standard Curves

****Normalized to in-well Amino Acid concentrations****

Nanoparticle Batch	HAuCl ₄ Added (g)	NaBH ₄ Added (g)	ABS ₅₇₀ / ABS ₄₆₀ at 0g/L Amino Acid	Tested Linear Range (g/L)	Slope Δ(ABS ₅₇₀ / ABS ₄₆₀) / Δ(g/L)	R ² Value
1	.0049	.0010	.5563	0.15 - 1.0	.4411	.9727
2	.0055	.0006	.6752	0.15 - 0.9	.3769	.9736
3	.0051	.0012	.8587	0.15 - 1.05	.2825	.9720
4	.0065	.0009	.6937	0.08 - 1.05	.5749	.9733
5	.0057	.0008	.6592	.013 - .652	.6915	.9743
6	0.005	0.008	.7758	.005 - 0.24	0.25	0.96

Table 1: Tabulated summary of various nanoparticle synthesis components and resulting linearity results.

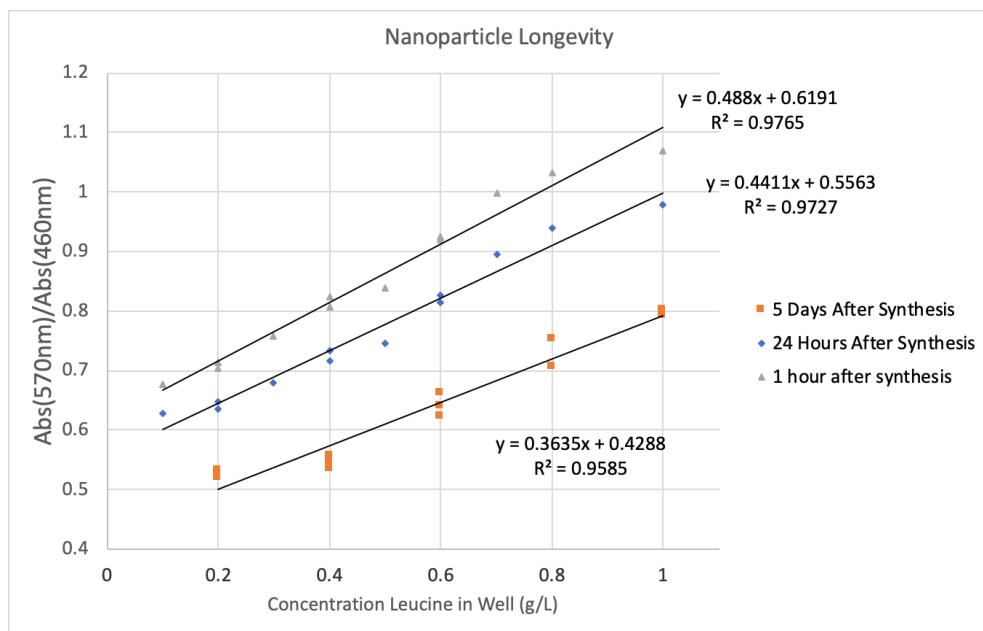


Figure 1: Nanoparticle standard amino acid curves measured over time.

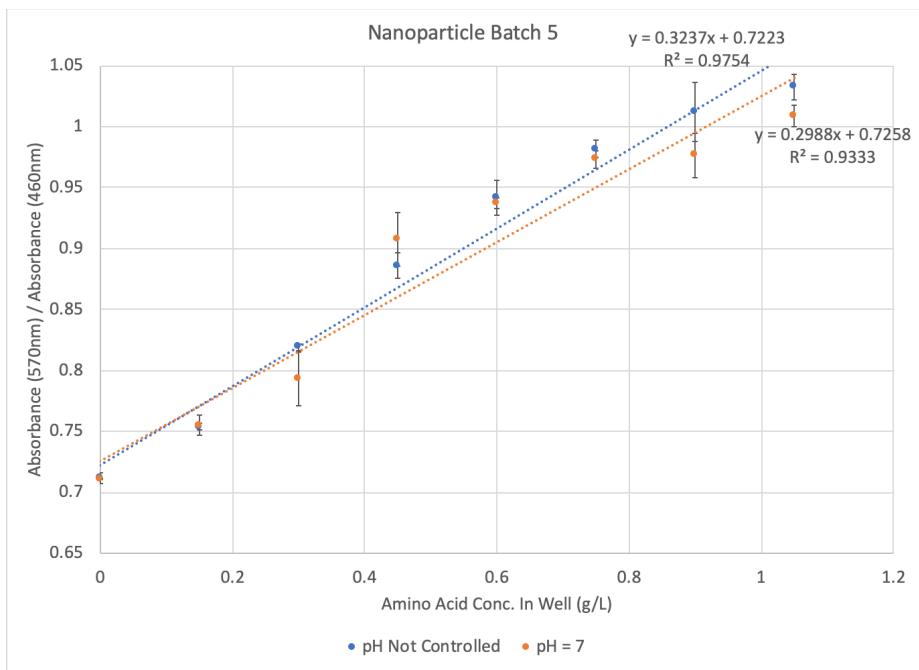


Figure 2: Nanoparticle standard amino acid curves measured with and without standard pH.

Appendix F

Statistics for Assay Development

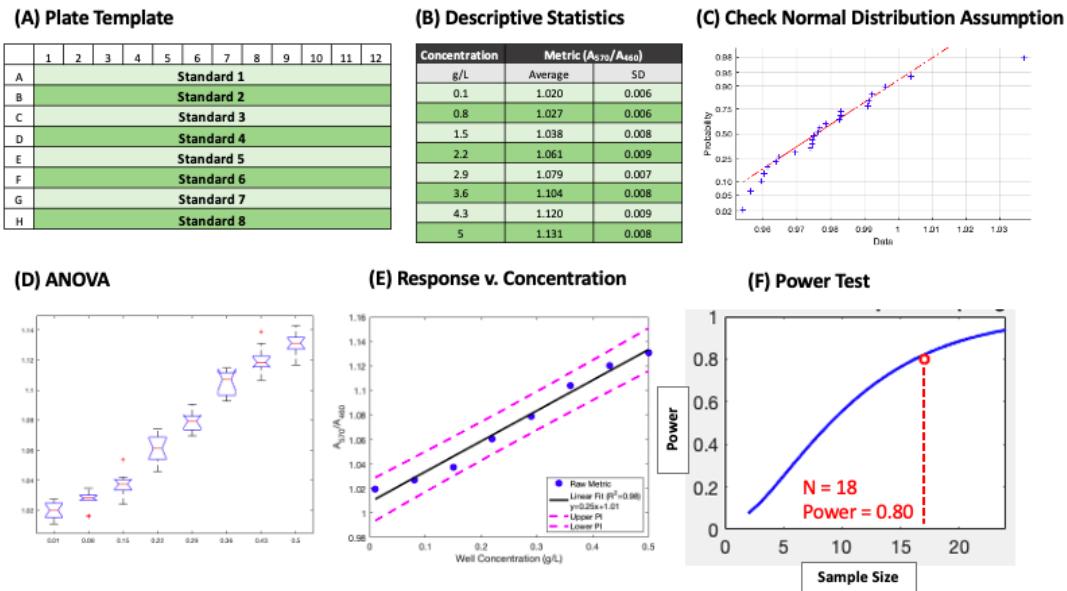


Figure 1: (A) Plate template for assay development testing, (B) Descriptive statistics for 8 standards, (C) Normal probability plot for one standard, shows visually that data follow a normal distribution and is further backed up quantitatively by the Anderson-Darling test, (D) Result of ANOVA test that shows concentration is a significant factor for A_{570}/A_{460} , (E) Response versus concentration curve with linear fit and prediction intervals ($\alpha = 0.05$), (F) Results of power test for one standard, in this case a sample size of 18 is needed to achieve a power of 0.80)

Standard Concentration (g/L)	Sample Size Needed For:	
	Precision: 10% Power = 0.80	Precision: 0.25 g/L Power = 0.80
0.1	4131	9
0.8	81	11
1.5	35	14
2.2	23	18
2.9	9	12
3.6	10	17
4.3	8	18
5	6	17
Maximum:		18
Recommended:		24

Table 1: Sample size required to achieve a Power of 0.80 for two levels of accuracy

Appendix G

MIP Washing & Reintroduction Experiments: Adsorption/Resorption Quantification

Purpose: The NAA was used to quantify aqueous leucine resorption during MIP post-synthesis washing and adsorption during template-reintroduction.

MIP Post-Synthesis Washing:

Unfortunately, the standard curve prepared the day of testing resulted in a nonlinear curve ($R^2 = 0.13$). Using a curve prepared the day prior ($R^2 = 0.98$), which was created using the same standards and batch of nanoparticles, the sample ratio (1.14 ± 0.0175 for $N=96$) corresponds with about 5 g/L, which would indicate that all of the template had been removed. This finding is consistent with the team's discussions with Professor Belbruno, who indicated that he had success removing all of the template molecules from his MIP by soaking with water.

MIP Template Reintroduction:

Following the washing experiment, one plate of MIPs was treated with trichloro(1H,1H,2H,2H-perfluorooctyl)silane to create a hydrophobic film on top of the MIP, which the team investigated as a potential method for preventing interfering molecules in the fermentation broth from entering or sticking to the MIP. Another plate was left without the film for comparison. A sample reintroduction solution (4.3 g/L) of leucine and water was then added to all wells on both plates. The NAA was again used to determine the concentration of the solution in the wells after 30 minutes and 24 hours, this time to analyze the effectiveness of the reintroduction protocol. Standard curves were prepared successfully for each of the two tests ($R^2 = 0.96$ for 30-minute test, $R^2 = 0.87$ for 24-hour test) and used to estimate the concentration of the reintroduction solution. Results from this experiment are summarized in Figure G1. The experiment showed that about 50% of the amino acid had adsorbed into the MIP after 30 minutes, indicating that 24 hours is likely much longer than needed to achieve 100% adsorption. However, with the trichlorosilane film, template reintroduction is much slower, only achieving 33% adsorption after 24 hours. The film clearly hampers the adsorption of the template, which will have to be considered even if it successfully prevents most interfering molecules from entering the MIP from the broth.

		Estimated Concentration (g/L)	% Leu in MIP
30 MIN	No Film	2.00	53.8%
	Film	3.15	26.5%
24 HR	No Film	0.00	100.0%
	Film	2.88	33.1%

Table 1: Results from reintroduction experiment, starting with a 4.3 g/L reintroduction solution

Appendix H

MIP Adsorption of Leucine-Spiked DMC Broth Samples

Media	MIP Extract Conc. (g/L)	Broth Conc. (g/L)	Recovery % after 45 min
Seed Media	0.18	3.61	5.08%
Seed Media	0.18	1.20	15.12%
Seed Media	0.40	4.80	8.28%
Ultrafiltrate	0.20	3.61	5.51%
Ultrafiltrate	0.37	1.20	31.02%
Ultrafiltrate	0.29	4.80	5.95%

Table 1: Estimated concentration of extracted solution versus adsorption broth solution based on NAA standard curve in Figure 1 below (adsorption time 45 minutes; extraction time 45 minutes).

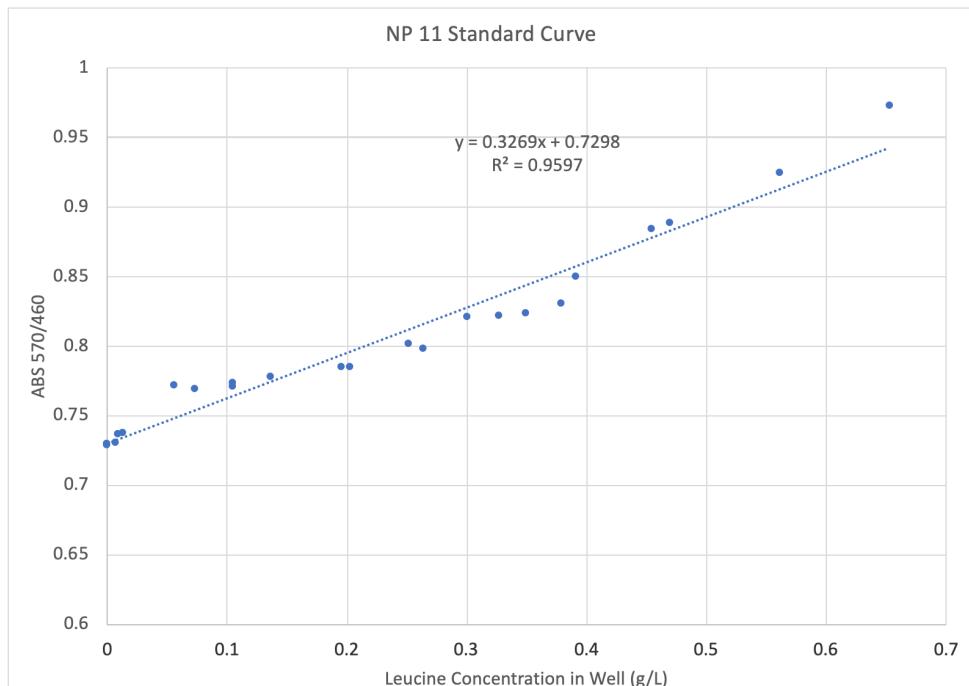


Figure 1: NAA standard curve.

Appendix I

Adaptability & Specificity Testing: MIP-NAA

MIP-NAA Adaptability

The team investigated whether the linear standard curves for leucine and water samples obtained using the MIP-NAA could be replicated using iso-leucine and water samples. This experiment was not to test whether the assay could distinguish between iso-leucine and leucine, but rather to see if the two analytes would cause a similar response in the nanoparticles that could be correlated with the metric A_{570}/A_{460} to estimate concentration. The standard curves for iso-leucine are included in Figure X1. The plot on the left shows the team's first attempt at creating an iso-leucine standard curve and shows that the amino acid causes more rapid agglomeration and change in the metric than does leucine, causing the curve to flatten quicker or saturate. The plot on the right shows a later attempt that successfully achieves linearity by diluting the samples and investigating a smaller range. It should be noted that the error bars are significantly larger at the lower range than at the higher one, which could be a challenge in implementing the MIP-NAA with iso-leucine. Using a lower concentration range to achieve a linear standard curve would have to be balanced with the likelihood that lower concentrations lead to larger error bars, perhaps due to greater inconsistencies in pipetting such small volumes into the wells.

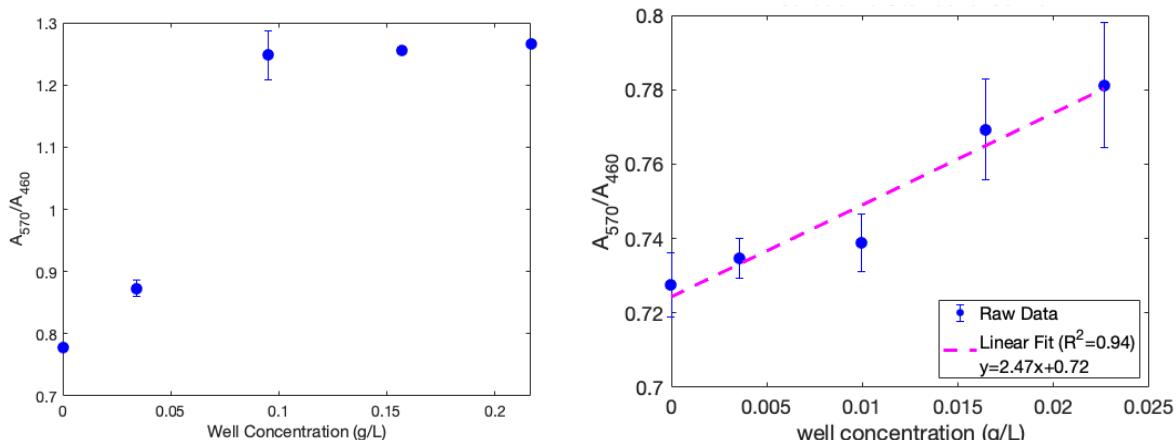


Figure 1: Iso-leucine standard curves obtained using MIP-NAA; (Left) First attempt results in early saturation of the curve (Right) Second attempt with lower concentration range results in linear curve, but larger error bars

MIP Assay Specificity & Adaptability

Because the initial iso-leucine standard curves obtained by the team were not linear (saturated too early), the team instead looked to investigate whether the average metric A_{570}/A_{460} was significantly different across replicates of a standard solution that sat in a well with an MIP versus one that did not. To do this, a two-sample t-test was performed to determine whether the independent random samples (A_{570}/A_{460} of well sample vs. metric of standard from vial) were

from normal distributions with equal means and equal but unknown variances. All results supported MIP specificity except the adsorption of iso-leucine into the iso-leucine MIP. The lack of adsorption of leucine into the iso-leucine MIP plate is also weakly supported due to a high standard deviation in the samples' A570/A460 ($\pm 10\%$).

Significant Adsorption Likely Occurred After 24 Hours		
MIP Template	Leucine Standard	Iso-Leucine Standard
Leucine	Y	N
Iso-leucine	N	Inconclusive

Table 1: Summary of conclusions supported by specificity experiment

MIP	Standard	Test		Control		Result of Hypothesis Test ($\alpha = 0.05$)	P-value
		Average A570/A460	SD	Average A570/A460	SD		
Leucine	Leucine	0.7322	0.0095	0.7924	0.008	1	6.29E-22
	Iso-leucine	1.3717	0.0092	1.3663	0.0096	0	0.7751
Iso-leucine	Leucine	0.96	0.1103	0.9961	0.0056	0	0.9444
	Iso-leucine	0.7693	0.0137	0.7802	0.031	0	0.3505

Table 2: Detailed results of two-sample t-test and descriptive statistics used to make conclusions about MIP specificity; Red indicates that the lack of iso-leucine adsorption into the iso-leucine MIP was unexpected and warrants further investigations

Appendix J

Specifications Table

	MIP-NAA	NAA	Coupled Enzyme Assay
1. Cost: ≤ \$1 per sample	✓ \$0.09/well	✓ \$0.04/well	\$3.15/well
2. Accuracy: ± 10% error in water	+ See Section 5.1; Linear fit of standard curve using MIP with leucine and water standards achieves $R^2 > 0.92$ but samples' metric (A_{570}/A_{460}) standard deviations range from 4-10%. <i>Further work is needed to analyze the level of accuracy to be expected with the full MIP-NAA.</i>	✓ For concentrations >3g/L, 10% accuracy (differentiation between concentration & concentration +10%) can be achieved with ≥9 replicates. Lower concentrations require more replicates to achieve the same accuracy.	- See Supplementary Materials 4. Lab Report 3; After troubleshooting, the leucine standard curve achieved an R^2 of 0.94 with samples' A_{540} standard deviations ranging from 1-13%.
3. Repeatability: Consistency between wells, plates, and analysts	+ See section 4.2.2 for uniform MIP synthesis repeatability. <i>Further work is needed to assess repeatability of MIP-NAA workflow results.</i>	✓ See section 4.4.3; Consistent linearity across batches of nanoparticles synthesized ($R^2 > 0.97$) but parameters of standard curve linear fit varied.	- See Supplementary Materials 4. Lab Report 3; The team found AAA Kit results for both the alanine standards provided and the leucine standard prepared to vary across multiple days of testing.
4. Dynamic Range: Linear range 0.1 – 5 g/L	+ See Sections 5.1 and 5.2; Response vs. concentration shifting was achieved for YPD broth. <i>Further work is needed to optimize protocol for DMC broth.</i>	✓ See Section 4.4.3; Linearity was consistent across 0.1-1 g/L (well concentration; this can be extended up to 14 g/L with greater dilution).	✓ The purchased assay kit was intended to work for concentrations within 0.01-0.13 g/L, but higher concentrations could be assessed with greater dilution.

5. Specificity: Assay attains 10x greater signal for analyte of interest than for interfering molecules	+	See <i>Section 5.3</i> and Appendix I; the MIP adsorbs 100% of leucine in a 4.3 g/L sample within 24 hours (likely less time) and insignificant amounts of iso-leucine for a solution of the same concentration and the same wait time.	-	The assay does not distinguish between the analyte of interest and interfering molecules; Interfering molecules, most critically carboxylic acids and amino acids that are not the analyte of interest, must be removed from the sample solution.	-	Similar to the NAA, enzymatic assays like the AAA Kit are not meant to distinguish between the analyte of interest and interfering elements.
6. Robustness: Ability to function in the presence of interfering molecules	+	See Supplementary Materials 4. Lab Report 7; MIP is not chemically changed when combined and allowed to sit with fermentation broth, but interfering molecules tend to stick to nylon-6 surface, causing errors in the NAA.	+	Assay is robust in moderate amounts of ammonium and excess amounts of glucose, but carboxylic acids and interfering amino acids cause critical error.	The AAA Kit is not intended to be robust in the presence of interfering molecules.	
7. Adaptability: Ability to be used across multiple analytes in DMC's pipeline	✓	See <i>Section 5.3</i> and Appendix I; Leucine and iso-leucine MIPs were synthesized and proof of concept of the NAA was achieved for both analytes.	✓	See <i>Section 5.3</i> and Appendix I; Proof concept of the NAA was achieved for both leucine and iso-leucine.	The AAA Kit was intended for quantification of L-amino acids.	
8. Time-to-Result: < 1 week /1000 samples	✓	See Supplementary Materials 3. Final Protocol – MIP-NAA; 11 Plates (>1000 samples) could be synthesized in tandem for less than 4 hours of hands-on time with one technician working with a multi-channel pipette; The NAA takes less than 25 minutes to use.	✓	See Supplementary Materials 3. Final Protocol – MIP-NAA; The NAA takes less than 25 minutes to use excluding the 30-minute assay incubation time.	✓	The AAA Kit is simple to use and should take less than 1 hour to analyze a plate of samples excluding the 90-minute assay incubation time.

* with 95% confidence at concentrations $\geq 3\text{ g/L}$ ($n \leq 9$)

+ shows significant promise from preliminary experimental results and literature review

- attempted, but didn't work

Table 1: Specifications table comparing MIP-NAA and NAA to the state-of-the-art.

Appendix K

Yearly MIP-NAA Reagents Amounts

Component	Reagent Amounts		
	Per plate	Per week	Per year
Nylon-6	0.3696 g	3.696 g	192.192 g
Leucine	0.0924 g	0.924 g	48.048g
Formic Acid	3.4086 mL	34.086 mL	1.431 L
Acetic Acid	0.5 mL	5.0 mL	260 mL
HAuCl₄	0.00096 g	0.0096 g	0.4992 g
NABH₄	0.0002112 g	0.002112 g	0.110 g
Perfluorooctyl trichlorosilane	0.192 mL	1.92 mL	99.84 mL

Table 1: Predicted annual total reagent amounts required by MIP-NAA protocols for the synthesis of 10 plates per week.

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