Separation Of Racemates using Self-Assembling Monolayers

A Major Qualifying Project
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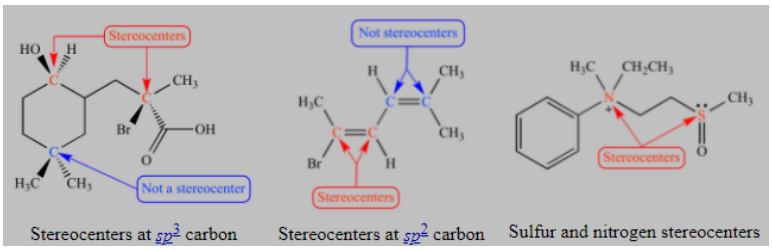
Abstract:

This Major-Qualifying Project examined the potential of using enzymes in a continuous flow process using self-assembly scaffolds.The first recorded observations of differences in the biological actions of stereoisomers was in 1886. Since then, there have been many chiral drugs identified to have adverse side effects due to an enantiomer in a drug and/or human pharmacotherapies that cannot be easily separated from its racemate. I explored the different methods of separating racemates and found the best scaffold to covalently bond self-assembling monolayers (SAM) with the enzyme carboxylesterase naproxen (CNP). The final design is a proposal for how we can develop this idea with these parts.

Introduction:



Two challenges in chemical production are the separation of racemic mixtures and the development of a continuous flow process. Racemic mixtures come about because normal chemical manufacturing techniques produce an even distribution of two molecules with different chiral centers, that is a carbon atom which can have up to four chemical bonds organized in different combinations. In the first figure from UCLA's chemistry, we can see the difference between chiral and non chiral centers. Racemic mixtures come in a lot of molecule configurations but they always are attached to at least three different groups exhibiting three different kinds of bonds. This makes distinguishing between two orientations very difficult, so there are few options to separate them during production. Continuous flow processes are a challenge because many chemicals production steps were developed as batch steps. One of the key contributions chemical engineering has made to process engineering is the development of continuous flow reactors, although these are not yet used everywhere.

The promise of biotechnology is that enzymes can be chiral specific - they produce only one enantiomer. Therefore, using enzymes to produce or separate chiral molecules is a promising strategy. There have been a limited number of techniques viable to separate enantiomers, and even less at biological conditions. The limited and unique conditions make the processes much more costly and they are usually done in batch processes. So, while the proteins are easy to produce, they are difficult to maintain and make the chemistry more complicated. This is especially true with synthetic molecules with stereocenters due to most of them being developed at very high or very low temperatures where enzymes are ineffective. However, there have been techniques experimented with to improve stabilization of the stereoselective enzyme, including

self-assembling monolayers (SAM), which is the focus of this paper. Our scope is to determine if using a SAM as a scaffold is viable in a continuous flow process and present a potential design that this kind of stabilization would be used in. In this work, I use kinetics to model the performance of a possible immobilized enzyme flow reactor for the separation of a racemic mixture of naproxen. I also describe how one might build such an immobilized enzyme structure using self-assembling monolayers (SAMs) and enzyme produced synthetically from the bacterium *Bacillus subtilis*.

Background:

The Importance of Chirality:

The first recorded observations of differences in the biological actions of stereoisomers are attributed to Piutt in 1886. Since then, there have been many chiral drugs identified to have adverse side effects due to an enantiomer in a drug and/or human pharmacotherapies that cannot be easily separated from its racemate. Some instances of chirality are noted in the field of pharmaceuticals and medicine by synthetic chemists, here are a few of the observed chiral molecules and the category they fall under:

(i) Analgesics: dextropropoxyphene, codeine, hydromorphone, oxycodone, morphine, methadone.

(ii) Anorectics: amphetamine, fenfluramine.

(iii) Antibiotics: amoxicillin, ampicillin,

(iv) Anticoagulants: warfarin, heparin, nicoumalone.

(v) Antihistamines: brompheniramine, chlorpheniramine, clemastine, promethazine

(vi) Anti-Parkinson agents: L-dopa.

(vii) Sedatives/hypnotics: phenobarbitone, glutethimide.

(viii) Anticonvulsants: Methyl phenobarbitone, methsuximide, phensuximide, ethosuximide.

(ix) Anti-inflammatory: ibuprofen, ketoprofen, fenoprofen, **naproxen**, sulindac

(x) Anaesthetics general: ketamine, methohexitone, etomidate

(xi) Sympathomimetics: ephedrine, pseudoephedrine, phenylpropanolamine.

(xii) Antidepressants: tranylcypromine.

(xiii) Anxiolytics: oxazepam, lorazepam.

(xiv) Cardiac: propranolol, labetalol, timolol, verapamil, disopyramide, mexiletine, tocainide.

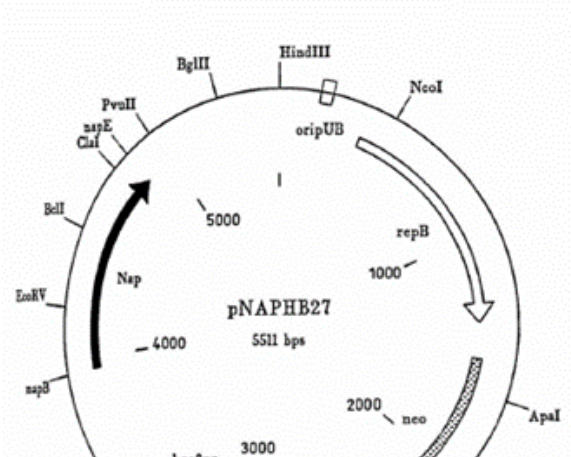
Logically, new studies in biochemistry were launched after these chiral molecules were observed and some of the racemates listed have been resolved by one method or another. This gives us a perspective on which enantiomers can be separated and which cannot. We will review some of the methods used later on.

Current Approaches to Racemic Mixture Separation:

Quantitative Crystallization Resolution:

In Ye, X. et al. looks at quantitative, selective crystallization that represents one of the most economical and convenient methods to provide large-scale optically pure chiral compounds (Ye, X. et al., 2019). Although significant development has been achieved since Pasteur's separation of sodium ammonium tartrate in 1848, this method is still fundamentally low efficient (low transformation ratio or high labor). An enantiomer-selective-magnetization strategy for quantitatively separating the crystals of conglomerates by using a kind of magnetic nano-splitter. This technique reached a purity of 99.2 ee% for R-crystals, 95.0 ee% for S-crystals, with a high separation yield of 95.1%. It has proven to be a great potential in developing chiral separation methods used on different scales.

This crystallization technique can allow us to separate more enantiomers but at an increasing cost. This technique can be effective but requires the addition of nano-splitters to come between the molecules to fully separate the growing crystal structure and allow for magnetic separation. The goal of my MQP is to remove the need for nano-splitter and magnetic separation and replace it with an enzyme to selectively separate one reactant into the desired molecule while keeping the other racemate in its original form.



Enzymatic Resolution:

In the 1994 edition of *Applied Microbiology and Biotechnology,* Quax W. J. et al. and their article titled *Development of a newBacillus carboxylesterase for use in the resolution of chiral*

drugs describes the resolution of a chiral solution of (R,S)-naproxen enantiomers with a carboxylesterase naproxen (NP). The organism used in this resolution is *Bacillus subtilis*, which can receive the cloning parts to produce an enzyme that is stereo-selective for the synthesis of the R-enantiomer of naxopen methyl ester. This enzyme takes the molecule the R-naproxen methyl ester and catalyzes it into the molecule S-naproxen.

The stereo-selective enzyme carboxylesterase naproxen (NP) was produced in the plasmid (pUB110) with the bleomycin resistance gene (*bleo)*, neomycin resistance gene (*neo*), replication protein (*repB*), origin of replication (*oripUB*), synthesis of second strain (*palU*), promoter (*hpa2pr/Hpa II*) (Zyprian and Matsura, 1986). The orientation of these in the plasmid can be seen in Figure 1. The carboxylesterase NP is an intracellular protein due to the lack of a signal peptide; a long hydrophobic region proceeds with some positively charged residues. It was found in Quax, W. J., et al. that the enzymes from *B. subtilis* strain 1A40/pNAPT-7 is superior to *Bacillus* Thail-8, at separating the enantiomers. This shows that genetic modifications have been applied to biosynthesis of this molecule and has shown to improve upon what *Bacillus* Thail-8 is capable of.

Immobilization of Enzymes on SAMs:

Once the basics of cloning with *B. subtilis* are proven through experimentation, we can begin to build characteristics around the science involved in genetically designing proteins. These sciences will involve a lot of kinetics and specific detailed characteristics involving chemical changes in the enzymes once they are bound to self-assembling monolayers (SAM) molecules. Much of the kinetics are dependent on how well the enzyme binds to the substrate, meaning we will most likely see slower or no enzymatic rates in proteins that have much of their active site covered or obscured. We will try to overcome this issue quickly with the immobilization of the desired enzyme on the SAM.

The ability of proteins to bind to SAM seems to be very good for “model” enzymes, but the enzyme we will be looking at is much more complicated and will need to perform more complex functions that need a more exact science. Each enzyme-racemate pair will need to have an analysis done in order to categorize and correctly create hypotheses about how the enzyme will interact with the SAM. The more we learn about how enzymes want to interact with SAM, the more we can predict the correct modifications for whole groups of enzymes that share similar characteristics. With these similar characteristics in mind, it is logical that we could use the same genetic modifications in order to improve SAM binding. This will add to the current library of genetically modified organisms we have surmounted thus far, and it will add an additional organism, with an additional tool kit for us to work with as the research is furthered.

The methods carried out by (Wadu‐Mesthrige, K. et al.) in their paper, *Immobilization of proteins on self‐assembled monolayers,* are a beginning to the start of developing methods to attach proteins, and have them remain active, in order to separate racemates. The methods used

to immobilize the proteins lysozyme (LYZ), bovine serum albumin (BSA), and immunoglobulin (IgG) are promising and in this paper we will try to incorporate a way for us to optimize the kinetics of the reaction.

Cloning with Bacillus Subtilis:

Bacillus subtilis is a common organism used in synthetic biology, especially in the pharmaceutical industry. The continuous innovation in the fields of vaccines and therapeutics requires the parallel innovation in the production of pharmaceutical proteins and the development of continuous flow processes to efficiently produce them in industry. When considering using organisms as cellular factories the host and conditions these proteins are produced in can affect the downstream product if efficiency is not coupled with quality. This even includes selecting the correct strain of the organism we wish to use. For example, there are several auxotrophic mutants of *B. subtilis*, which provides a system that can accept a variety of cloning parts through transformation. Being studied so heavily over the past few decades, *B. subtilis* has become a model organism for Gram-positive bacterium in the field of microbial biotechnology.

The first genome of *Bacillus subtilis (*strain 168) was sequenced twenty years ago, and a lot of research has been done surrounding this organism and its auxotrophic mutants. The reason that there is not consistent information on *B.* subtilis is that most information on *B. subtilis* is inferred through "the majority rule". This makes our job of replicating the science previously produced difficult. Meaning there is a lack of experimental data for scientists to look back on if the proof of concept experimentation does not work.[6] There have been experiments done to create a correction system, but there remains a lack of experimental validation for many of these methods. Errors continue to occur as seen by (Gilks, *et al., 2005)* which brings up the issue of the lack of sequence data to the forefront of the fields involving the genetic engineering of *B. subtilis* (Chang *et al.,* 2016).

What *B. subtilis* is most efficient at is the secretion of enzymes to degrade substrates not needed by the Gram-positive soil bacteria, which allows it to continuously survive in a changing environment and makes its genetics so valuable. Enzymes produced commercially by *B. subtilis* represent 60% of the industrial-enzyme market. [7] However, some of the issues with *B. subtilis* secretory pathway are poor targeting to the translocase, degradation of the secretory protein, and incorrect folding have all been revealed to severely hinder the *B. subtilis* secretion pathway. This mainly occurs with heterologous proteins, but the improvement of secretion of more complex proteins, will improve the overall science. The protein that is the focus of this report, carboxylesterase naproxen, is a homologous protein, which has a stereocenter that needs to remain highly conserved for the enzyme to remain active. [3]

Methodology:

Model:

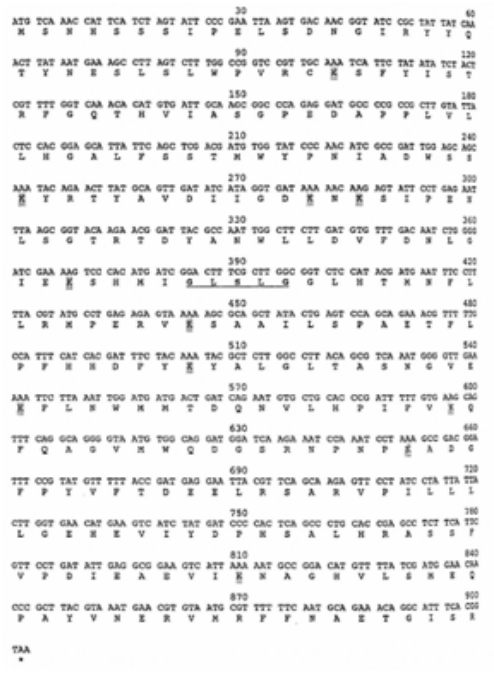
Using cellular factories is becoming more common yet more complex and the science behind separations of racemic mixtures is becoming less intuitive which makes it difficult for scientists to specialize and develop in this area. A useful analogy for the science I am proposing comes from the booklet called *Synthetic Biology – A Primer.* This depicts the complex work done by cellular organisms in an intuitive metaphor where cells are factories and enzymes are their machinery.

This analogy can be used to describe the science explained in the background information. We can use optical density to find the "factories density" in a solution that produces the right amount of "machinery per unit factory". This is really the number of enzymes produced by *B. subtilis* into the solution of the racemic mixtures. While this analogy is easy to follow, once we begin to look at the larger picture we can modify the analogy a little bit. In our process the production of the enzyme is only one part of a large system, which in this case is a continuous flow process using a packed-bed reactor. In this section, we will be discussing how to take what we have analyzed here and produce a theoretical process that can be used to produce a solution of S-naproxen and R-naproxen methyl ester.

With this metaphor in mind, we can examine an approach that is plausible on at least a pilot process level. Assuming most of the packed-bed can be designed without much issue, the main part we have to focus on is the reaction and making sure our enzyme (or machine part) is attached, available, and able to change our reactant into our product. This means designing the organism, *B. subtilis*, that "best" produces the enzyme with a vector of the "best" enzyme for scaffold attachment. Now, we have a well-developed enzyme to attach to a packed-bed of gold particles covered in SAM ending with a specific characteristic group. The amount of contact needed to push the reaction forward means the flow rate in and out needs to be low and the reactor should be well-stirred. Using the kinetic model from the paper, *Optimisation of stabilised Carboxylesterase NP for enantioselective hydrolysis of naproxen methyl ester,* we can gather reaction constants and reaction rate equations to determine flow rates and reactor size.

Discussion:

Feasibility of Enzyme Production and Design:

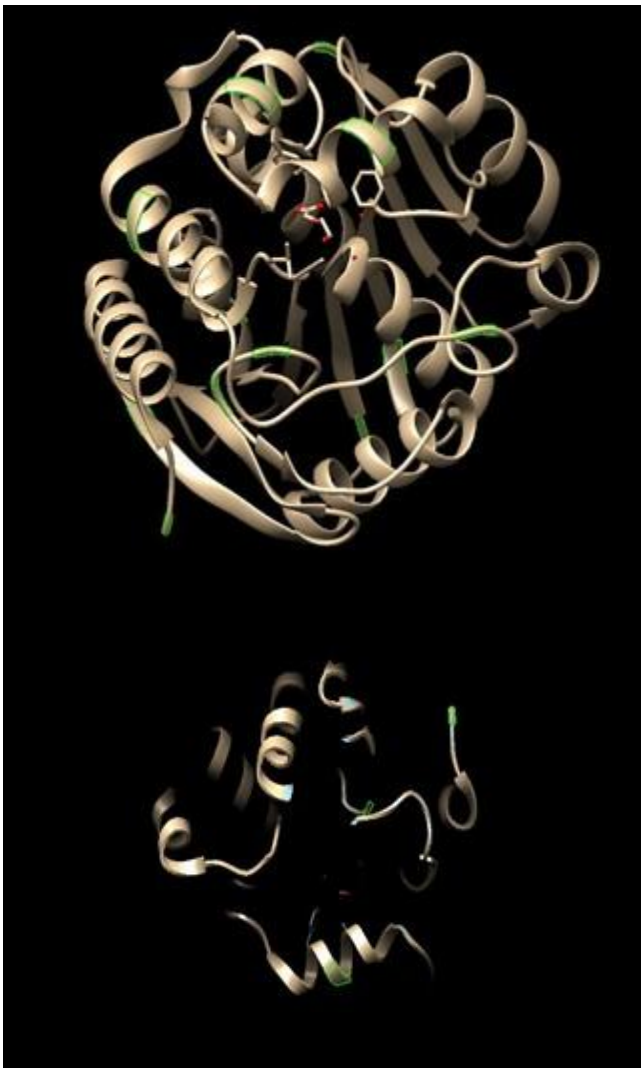


My project begins with the production of a carboxylesterase naproxen (NP), that resolves the chirality of the (R,S)-methyl ester naproxen to S-methyl ester naproxen and S-naproxen. [3] The genetic engineering of (Quax *et al.*, 2000) is the best place to start in order to replicate their results of producing the carboxylesterase NP and stabilizing it on a SAM. The genetic sequence of the carboxylesterase NP can be seen in Figure 2.[3] This comes from the single open reading frame in the insert of pNAPT-2.

Using this base sequence, we can alter it to produce a more efficient protein for our application. Once the desired sequence of the enzyme is determined, the optimal cloning process needs to be designed. There are many ways that we may produce this enzyme, but there are parts that are consistent throughout most genetic engineering in *B. subtilis*, such as the promoter from *Staphylococcus aureus,* HpaII (hpa2pr). This promoter is found previously in (Qua *et al,* 2000) to produce the *nap* gene by modifying the plasmid pUB110, a shuttle vector for plasmids in *E. coli* and *B. subtilis*, to create their own unique plasmid that produced the carboxylesterase NP enzyme named pNAPHB27. Replication was done using *oripUB* for the origin of replication of the first strand and *palU* as the origin of synthesis for the second strand. The antibiotic resistance genes, which were used for the selection of transformed *E. coli* and *B. subtilis,* were the *neo* (neomycin resistance) gene and the *bleo* (bleomycin resistance) gene, respectively. The restriction map of pNAPHB27 can be seen in the first figure. This details how our laboratory can replicate pNAPHB27, in order to prove the conceptual pieces of their experiment, while strengthening the data in literature surrounding *B. subtilis.* There is no mention of a terminator being used in (Quax *et al.,* 2000), but there is evidence to support that using a transcriptional terminator will help make a more stable carboxylesterase naproxen and in our design we will be using a terminator to see if this helps us produce the enzyme more efficiently.

With these parts, I believe it is very probable that the enzyme produced from them will be at least as sufficient as those used in the studies mentioned through this paper.

Feasibility of SAM immobilization:



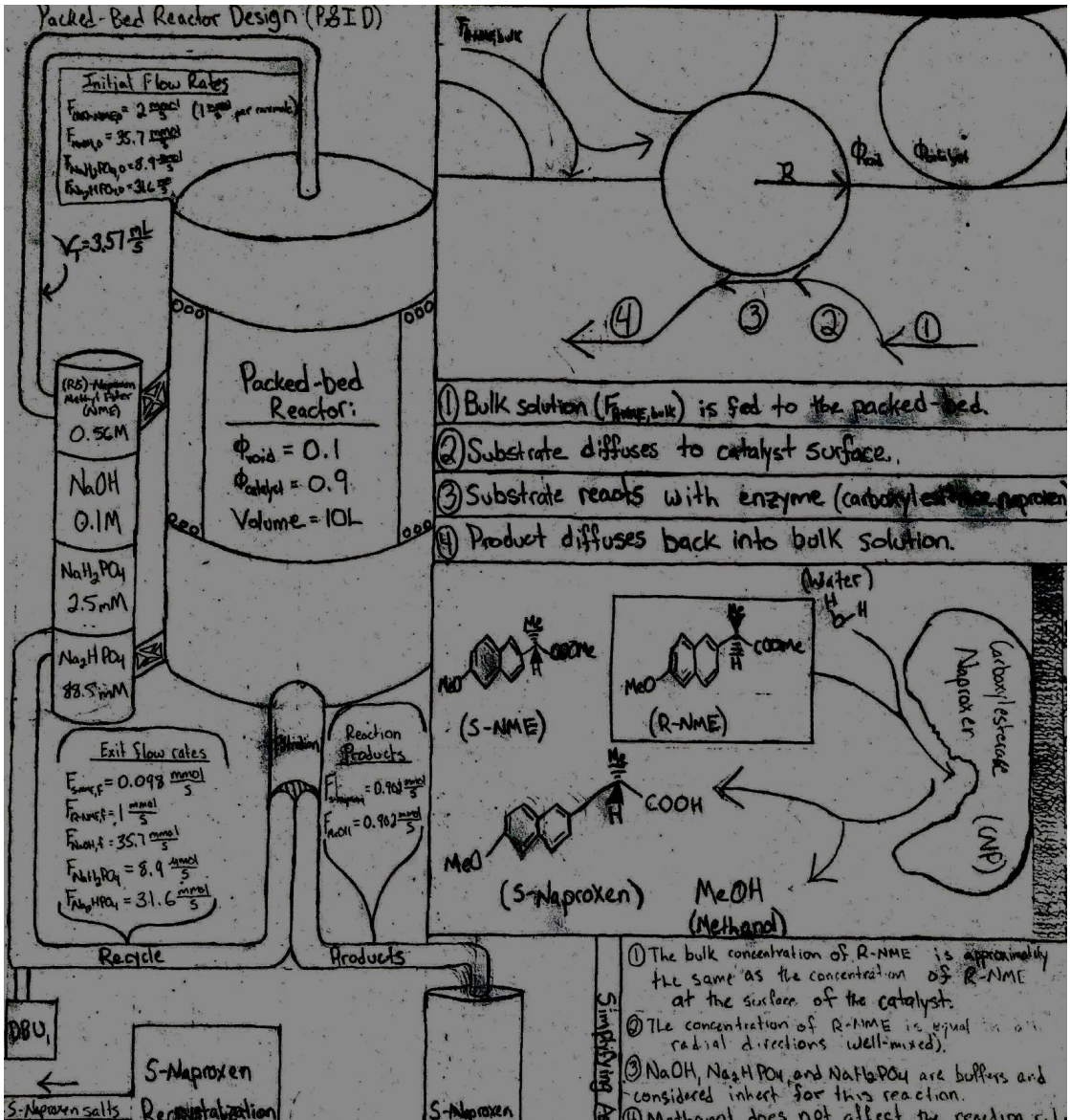
Potentially, there will need to be genetic engineering to conform the biosynthesized protein to what chemical parameters are needed to accomplish what I aim to do. Using the National Institute for Health (NIH) databases, the carboxylesterase of *B. subtilis* was found on the BLAST database. There is a conserved amino acid triad of a serine, glutamic acid, and histidine that is seen throughout all genes that encode for a carboxylesterase. This gives us a good idea for the essential domains and conserved domains of this enzyme.

This idea of genetically modifying the enzyme will most likely need to be applied in order to attach the enzyme to a SAM. What we need to answer first, is whether the enzyme will be active while bonded to the SAM. This is a concern, because the methodology for attaching enzymes to SAM uses the NH_3 bond from the N-terminus to bind itself to the SAM's end atom, which is flexible, but this could affect the electrochemistry of the active site, as well as block the active site from being available to the substrate. If this is the case, we must eventually design an enzyme that will allow it to attach to the SAM with the correct orientation of the active site; however, this is further than the scope of my project but it is a point to consider. I hypothesize that this could be overcome with the addition of a tail to the N-terminus, allowing all parts of the enzyme to be exposed. In order to begin developing these hypotheses we must first look at the structure of CENP.

Using the software, Chimera, I was able to look at the structure of carboxylesterase NP from a 3-D perspective. It also allowed me to highlight the specific NH_3^+ atoms in the enzyme; this distinctly shows the N-terminus of the enzyme relative to where the molecule binds to the enzyme. As seen in Figure 3, there are other NH_3^+ atoms relatively close to the active site, but they are not as exposed as the N-terminus. The NP can be seen by its red oxygens highlighted, and the N-terminus is in the bottom left-hand corner of the image. Chimera also allows for a focused view where only certain planar sections are seen, and relative distance is much easier to develop once we remove all the enzymes except for the face of the activation site. In Figure 3,we can now see clearly that the N-terminus is on the same side as the activation site, which could prove to be a problem.

Overall, it looks like experimentation is needed in order to determine many parameters that need to be tweaked. However, there are plenty of previous experiments that suggest that if the conditions are similar enough, the concept of attaching the enzymes to the catalyst is definitely feasible.

Reactor Model:



Process Overview:

For this project, once the necessary protocols have been determined for proper production of our carboxylesterase naproxen, we can begin to design a continuous flow reactor for immobilized enzymes to catalyze their reaction. In order to do this we have to determine a few intrinsic characteristics of our reactor such as: the type of reactor we are going to use, the size, shape, and surface morphology of the material inside the reactor, and determining the most efficient way to run the reactor based on calculations. For the reactor we are going to be designing, it was determined that it would be best to use a pack-bed reactor due to the slow reaction rate of the substrate with the enzyme and the ability to have a high amount of surface area for the enzyme to attach itself. For the material inside the reactor it makes the most sense to use spherical gold nanoparticles with self-assembling monolayer (SAM) attached to the surface. This material will allow us to maximize the surface area to volume ratio and allow for more kinetic interactions between the enzyme and the substrate. Part of the process in determining the best way to run our reactor is how long it takes the enzyme to attach and what amount of enzyme is able to attach to the SAM. While this is important, it will not be the focus of our calculations as there is a lot of experimentation needed to be done in order to determine this parameter accurately. For now, we will assume the amount of enzyme on the surface of the Au-SAM complex will be 90 percent of the nanoparticle surface area, 60 percent of these enzymes will be considered bioavailable.

Start-up (enzyme attachment):

For this design we have decided to use the packed-bed reactor (PBR) model. This design will consist of a start-up process for immobilization of our enzyme (carboxylesterase NP) to the surface of our Au-SAM nanosphere complexes. This will model a continuously-stirred batch reactor as the packed-bed will be filled with a solution of our enzyme which will be at a pH of its isoelectric point (IEP) which gives the high yield of immobilized enzymes, which is around 90 percent surface area coverage (Immobilization of Proteins on Self-Assembled Monolayers). The IEP of our enzyme has been determined to be 5.3 and for now we will assume for simplicity that we are able to achieve 90 percent coverage of our Au-SAM complexes. The start-up will take approximately one hour, at which time the reactor will be drained of the solution and the remaining concentration will be measured to approximate the average amount of enzyme per nanosphere. While 90 percent is a good amount of immobilized enzyme there is a lot that happens when the enzyme interacts with the SAM. There are two main ways to immobilize a protein on a strategically-terminated SAM:

1. **Immobilization by physical interaction** (i.e. Van der Waals forces): which can be done with three different SAMs each terminated with a hydrophobic group, a non-ionizable hydrophilic group, and an ionizable hydrophilic group. These each have their own

benefits but it was found that the more charged the characteristic group is the more sensitive it is to its IEP but the immobilization yield is much higher than less charged groups when it is at the proper pH. IEPs of proteins vary widely and makes it difficult to assume an IEP for our enzyme, for now we will assume the IEP is around the same pH as when it has the highest kinetic activity which is around a pH of 7.5. The one benefit of using physical interactions instead of covalent bonds is that it scarcely effects the folding of the protein and leaves a majority of the enzymes bioavailable to the substrate, however, these interactions are not as stable and leaves the possibility of the enzyme detaching when it reacts with the substrate.

2. **Immobilization by covalent bond:** It is known that the primary amine group of lysine residues in proteins reacts with aldehydes (Baker et al. 1998). Using aldehyde-terminated SAMs, we are able to use this covalent bond to immobilize our enzyme on the surface. There are a lot of lysine groups in carboxylesterase NP and each one could change the morphology of our enzyme in a different way when reacted with the aldehyde. The average amount of bioactive enzyme after the covalent bond is 60 percent (Immobilization of Proteins on Self-Assembled Monolayers). However, the benefit of having these bonds is that the enzyme is much more stable and suitable now for long-term use, which can be very important in industrial application.

For our design we are going to use aldehyde-terminated SAMs due to the longevity and stability it gives to the enzymes.

Steady-state:

Once the reactor has been drained, the solution of our racemic substrate, naproxen methyl ester (R/S-NME) will begin to fill the tank. The initial flow rate ($F_{NME,o}$) will be determined based on the reaction rate. The reaction rate is calculated with the equation:

$$-r = \frac{kcat\ (Cnp)\ (Cnme)}{Km + Cnme}$$

Where r is the amount of moles of substrate reacted per second, k_{cat} is the turnover number or catalytic constant, C_{NP} is the concentration of our enzyme, C_{nme} is the concentration of the substrate, and K_M is the Michaelis-Menten constant. These constants were determined in the paper, *Optimisation of stabilised Carboxylesterase NP for enantioselective hydrolysis of naproxen methyl ester*, and determined and put in the table below:

Table 1.

K_{cat} (s^{-1})	C_{NP} (M)	C_{NME} (M)	K_M (M)	-r (mol/s)
4.9	$6.79*10^{-9}$	$2.174*10^{-3}$	$8.7*10^{-5}$	$3.2*10^{-8}$

In their experiment, they used the carboxylesterase NP in solution and not attached to SAM by covalent bonds. This means that we have to try and mimic the enzyme concentration as best we can. To do this we must determine the amount of catalyst per gold sphere. The gold beads are assumed to have a diameter of 2 mm, in a 10 L reactor this will fit approximately $2.4*10^5$ beads and have a total SAM-Au surface area of $3*10^6$ mm^2 but only about 90 percent of this will be covered, leaving $2.7*10^6$ mm^2 available for active enzymes. The dimension of the carboxylesterase NP is about 5 x 5 x 5 nm^3 meaning every enzyme takes up about 25 nm^2 of surface area. Converting nanometers squared to millimeters squared and dividing the total available surface area by the surface area per enzyme and we get the approximate number of enzyme in our reactor, $1.08*10^{17}$ total enzymes. However, this calculation does not take into account the space inbetween the catalytic beads (i.e. the void fraction, Φ_v).

The void fraction is the fraction of the packed-bed that is free space available for the substrate to move between our catalysts; this volume has a concentration that is equal to the bulk concentration of the feed. Fortunately, for our process the reaction is the rate limiting step. Therefore, we consider the concentration at the surface of our catalyst equal to that of our bulk concentration. Once we have the substrate concentration at the surface we need to find a new concentration of our enzyme that is bioavailable, or the effective concentration ($C_{NP,\,eff}$). The effective concentration is the amount of enzyme per unit surface area per gold bead. Now we must set an arbitrary void fraction that makes sense for our system. Since our reaction is dependent on getting as much substrate to collide with as many enzymes as possible, the more condensed the beads are the higher the yield of the product. While this may slow down the flow rate we use for the feed, the reaction rate is also fairly slow and a slower flow rate will allow more time for it to go to product. With this being said a void fraction of about 0.1 would still allow the feed to flow freely through the packed-bed, yet keep a high concentration of enzyme available to the substrate.

Reaction rate equations:

Now that we have determined the void fraction we can calculate $C_{NP,\,eff}$ as well as-$r_{s,eff}$, -$r'_{s,eff}$ -$r''_{s,eff}$. These are all using the effective concentration, $C_{NP,eff}$. Using Equation 1 from earlier we can calculate a -$r_{s,eff}$, which is $7.61*10^{-7}$ $\frac{M}{s}$(See Appendix for calculations). Now we have to use,

$$-r_{s,eff} = (-r'_{s,eff})(C_{NP,eff})$$

To find -$r'_{s,eff}$, which is calculated to be 4.76 $\frac{mol\ P}{mol\ E*s}$ (See Appendix for calculations)

And lastly the equation for -r″ is,

$$-r''Ac = -r'$$

Where $-r'_{s,eff}$ is the rate of reaction per mole catalyst (mol P/(mol-cat)(s)) and A_c is the surface area of the catalyst, $1.5*10^{7}\ \frac{m2}{mol\ E}$. It is calculated to be $3.17*10^{-7}\ \frac{mol\ P}{m2*s}$ (See Appendix for calculations).

Reactor size and initial flow rate:

Lastly, we must determine the size and initial flow rate. We are going to set the volume of the reactor to 10 L for the purpose of a pilot process and this paper. Therefore, we need to use the equation:

$$N_{catalyst} = \frac{1}{2} * F_{r\text{-}nme,o} \int_{0}^{0.9} \frac{dx}{-r'r-nme}$$

Rearranging this to solve for flow rate we get $1.55 * 10^{-6}$ mol R-NME per second (Calculations in appendix)

Conclusion:

There are many ways to go about creating a continuous flow process but to figure out which ones are viable there must be research done. That is what this paper attempts to do by outlining how one would go about doing research for a SAM-enzymatic catalyst system. With the proper adjustments, this could even be used for other systems that use different scaffolds and different enzymes. Overall, we believe that this is a viable process that has merit and potential if it were to be developed. As science develops, there needs to be new and innovative ways to progress the already advanced sciences and hopefully this can be a start to this further development.

Sources:

- 1. Arnold H. Beckett, 1990
- 2. Ye, X. et al., 2019
- 3. Quax W. J. et al., 2000
- 4. Wadu‐Mesthrige, K. et al.

- 5. Mentel M. et al.
- 6. Borriss, R. et al. https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/1751-7915.13043
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Calculations:

Calculations for the effective enzyme concentration ($C_{CNP,eff}$), $r_{NME,\ eff}$, $r'_{NME,\ eff}$, $r''_{NME,\ eff}$, and $F_{R\text{-}NME,O}$.

Index:

1. $C_{CNP,eff}$ - Effective concentration of enzymes in the packed-bed reactor
2. $r_{NME,\ eff}$ - Effective reaction rate ($\frac{mol}{L*s}$).
3. $r'_{NME,\ eff}$ - Effective reaction rate of the ratio between moles of substrate to moles of enzyme per unit time ($\frac{mol\ R-NME}{mol\ E*s}$).
4. $r''_{NME,\ eff}$ - Effective reaction rate of the moles of substrate per unit time per unit area ($\frac{mol\ R-NME}{m2*s}$).
5. SA_{cat} - Surface area per gold sphere
6. SA_{enzyme} - Surface area per enzyme
7. Φ_{cat} - fraction of volume that contains catalysts
8. Φ_{void} - fraction of volume unoccupied by catalysts
9. V_r - Volume of the packed-bed reactor
10. N_A - Avagadro's number

Reaction Rate Constants:

11. $K_{cat} = 4.9\ s^{-1}$
12. $K_M = 8.7 * 10^{-5}$ M
13. $C_{R\text{-}NME} = 0.28$ M

$C_{CNP,eff}$:

$$C_{CNP,eff} = \frac{(\frac{SAcat * \Phi e}{SAe}) * (\frac{Vr * \Phi cat}{Vcat})}{(Na * Vr)}$$

$$= \left[\frac{\left(\frac{(12.57e{-}6\ m2)*(0.9)}{2.5e{-}17}\right)*\left(\frac{(10\ L)*(0.9)}{4.19e{-}6\ \frac{l}{cat}}\right)}{\left(6.022e23\ \frac{molecule}{mol}\right)*(10\ L)}\right]$$

$$\underline{C_{CNP,eff} = 1.614*10^{-7}\ M}$$

$r_{NME,\ eff}$:

$$r_{NME,\ eff} = \frac{Kcat * Ccnp,eff * Cr{-}nme}{Km + Cr{-}nme}$$

$$= \frac{(4.9\ s{-}1)*(1.614e{-}7\ M)*(0.28M)}{(8.7e{-}5\ M) + (0.28\ M)}$$

$$\underline{r_{NME,\ eff} = 7.9 * 10^{-7} - \frac{mol}{L*s}}$$

$r'_{NME,\ eff}$:

$$r'_{NME,\ eff} = \frac{r\ nme,eff}{Ccnp,eff}$$

$$\underline{r'_{NME,\ eff} = 4.9\ \frac{mol\ r{-}nme}{mol\ E*s}}$$

$r''_{NME,\ eff}$:

$$r''_{NME,\ eff} = \frac{r'NME,eff}{Sa}$$

$$Sa = 1.5 * 10^{7}\ \frac{m2}{mol\ CNP}$$

$$\underline{r''_{NME,\ eff} = 3.27 * 10^{-7} - \frac{mol\ R{-}NME}{m2*s}}$$

$F_{R\text{-}NME,0}$:

$$N_{catalyst} = \frac{1}{2} * F_{r\text{-}nme,o} \int_{0}^{0.9} \frac{dx}{-r'r{-}nme}$$

$$F_{R\text{-}NME,O} = 1.55 * 10^{-6}\ \frac{mol\ r{-}nme}{s}$$