CAPILLARY-BASED MICROREACTOR FOR SCREENING PEPTIDE CATALYSTS IN THE ALDOL REACTION

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In recent years, the concept of miniaturization has been applied to many areas of chemistry, one of which is the synthesis arena with the development of new microreactor technology. Compared with conventional scale vessels, microreactors are advantageous in many aspects, such as faster mixing, better heat transfer, minimal reagent use and great safety. However, the technology of microreactors is still immature and most organic reactions are simply carried out based on the existing concept and setup of 'lab-on-a-chip'. The analysis-oriented chips are always incompatible with demands of organic synthesis and so the development of synthesis-oriented microreactors is of great need.

Our laboratory developed a novel capillary-based reactor system that was specific for high-throughput synthesis and screening. The computer-controlled reactor system integrated standard HPLC apparatus (autosampler, pump), fused-silica capillaries and GC in which separate zones of reactants and catalysts can be combined and loaded serially into a single reactor capillary, reacted in parallel and ejected serially for online GC analysis.

One of the applications of our microreactor was to study peptide-catalyzed aldol reaction. We chose a model aldol reaction with benzaldehyde and acetone substrates and known catalyst L-proline. Chiral GC separation conditions were optimized for determination of chiral aldol products. The optimum reaction conditions were 10 mol% L-proline catalyst, DMSO and acetone 1:1 (v/v), room temperature and 4 hr reaction time. A little amount of acetic acid was

added to increase L-proline solubility in organic solvents. Several peptides were preliminarily screened in the microreactor. Unfortunately, all of them showed poor activities.

The next step is to keep screening active peptide catalysts by our microreactor. Besides, novel solvents will be studied to further increase product yield and selectivity. The microreactor will also be optimized to increase its throughput and efficiency. The optimization process will be based on the combination of mathematical calculation (Mathcad software) and experiments. Moreover, the design of the microreactor will be improved in some units to make the system capable of accommodating more types of reactions such as multi-step reactions, gas-phase reactions or gas/liquid multiphase reactions.

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PREFACE

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1.0 INTRODUCTION

1.1 MOTIVATION

1.1.1 μTAS

Over the past few decades, miniaturized total analytical systems (μTAS), often called "lab-on-a-chip", or microfluidic devices, have been growing rapidly in a variety of fields including DNA analysis, drug discovery, pharmaceutical screening and medical diagnostics.[1-5]

Optimally, μTAS would carry out a complete analysis process that includes sampling, sample pretreatment, separation, detection and data processing in a fully automated and integrated fashion.[3] The most popular platform of μTAS is on the chip which is originally developed in the microelectronics industry. The microchannels (several to hundred micron in diameters), mixers, reservoirs, detectors are always integrated on chip to perform whole stages of separation and analysis.

Normally, chip can be made from many kinds of materials such as silicon, glass, quartz, metals and polymeric materials, among which glass and silicon are the most popular materials. Depending on the special properties of construction materials, there are a range of established microfabrication techniques available, for example, the combination of photolithography, wet

etching and bonding processes has been widely used to fabricate various designs of channel networks on glass chips.[6]

Electrokinetic or hydrodynamic pumping is generally used to motivate fluid flow through chip channels. Electroosmotic flow (EOF) offers a very convenient pumping mechanism for fluid manipulation with minimal hydrodynamic dispersion.[7-11] Electrodes are placed in the appropriate reserviors to which specific voltage sequences can be delivered under automated computer control. No external pumps or moving parts are needed, which make EOF easier to operate if more complex channel structures are integrated. However, pressure-driven flow (PD) also exploits conventional or microscale pumps to maneuver solutions around the channel network.[2, 12-15] There are several important theoretical advantages of PD over EOF pumping: (i) the control of flow velocity is more accurate by PD than EOF because PD is independent of the pH, electrolyte concentration, wall surface material, adsorption of molecules on the wall, composition of sample matrix, etc. (ii) PD has wider range of solvent selection. EOF has to pump solvents with some level of polarity, which limits its application in pumping organic non-polar liquids. (iii) When electrochemical detection method is preferred, there would be little interference between the force field needed for flow propulsion and the electrical fields needed for detection. (iv) PD offers a broader choice for chip materials too. It is necessary that the channel substrate materials should have a very low electrical conductivity for EOF flow, then, some popular chip materials like silicon have to be excluded by EOF flow.[16]

Research into μTAS or other miniaturization technologies are primarily driven by the desire to increase throughput and automation, while reduce the chemical consumption and lower the cost. The early application of microfluidic devices in 1990s is focused on separations techniques, especially by coupling on-chip capillary electrophoresis (CE) for fast separation of biological

molecules such as DNA.[17] However, with the development of delicate fabrication and integration techniques, μTAS systems have extended applications into broader areas. Complex channel designs, uniform tunnel structures and versatile online or offline detectors such as fluorescence or mass spectrometer make it possible to carry out more complicated experiments on chip.[2] One of the most important applications of μTAS is the integrated DNA analysis system used for ultra-fast DNA characterization. Through silicon and glass chip microfabrication, polymerase chain reactions (PCR) can take place in a temperature controlled reaction chamber including heaters and sensors which are integrated on chip with a sample mixing and positioning system, an electrophoresis separation unit, and an integrated fluorescence detection system. Only micro- or nanoliter volumes of DNA sample is needed and a whole analysis process normally takes less than several minutes.[3, 18] Other biological applications such as medical diagnostics are also undergoing rapid development now.[19]

1.1.2 Microreactor

While most applications in this area have been directed toward analysis, more recently, some have been directed toward synthesis. A number of research groups have focused on developing new microreactor technology originated from the existing μTAS concept. Several excellent reviews written from different perspectives have summarized the achievements of the integration of μTAS and microreactor technologies.[20-28]

So what are the advantages of microreactors over conventional batch vessels? The general design of microreactors is derived from μTAS systems, which consists mainly of two formats, the predominant chip-based microreactors with etched channels on a planar surface or less developed capillary-based microreactors utilizing commercially micro-liter capillaries as

channels. The fluid pumping in the microreactors is in the same way to μTAS, through the electroosmotic flow or pressure-driven flow.

Basically, the fluid transport inside the microchannels is quite different from macroscale fluidics. Reynolds number is normally used to quantify the degree of flow turbulence, which is dependant on fluid viscosity, density, velocity and channel diameter. In macroscale environment where Reynolds number is high due to large dimension scale, turbulence is dominant. However, in microscale dimension, turbulence is almost unattainable and laminar flow is dominant with a characteristic of mass transfer limited by molecular diffusion.[29]

The fundamental mixing process of liquids generally involves two steps: firstly, turbulence creates a perfect-dispersed heterogeneous mixture and secondly, molecules located in adjacent regions diffuse to make a homogeneous mixture. Thus, in traditional setup, mechanical stirring is mostly indispensable for creation of strong turbulence to bring reagent molecules close enough into diffusion limited dimensions, and then molecules can diffuse rapidly to form a homogeneous reaction solution. However, the time-scale of bulk stirring is much longer than that of diffusion. If the dimension of vessels is decreased to a certain level such as micron diameters where the diffusion rate is significant, the diffusive mixing would be so rapid that reaction time might be limited only by inherent reaction kinetics rather than the process taken for homogenizing reagents.[22] In practical terms, reactions performed in microreactors may produce comparable or higher yields within seconds/minutes in contrast to hours/days in bulk reactions. Greenway *et al*[30] studied the synthesis of 4-cyanobiphenyl at room temperature in a flow injection microreactor with a product yield of 67.7% within 25 seconds, while under the same conditions, the conventional batch synthesis can only give 10% yield with reaction time up to 8 hours. Actually, there is no difference for the chemistry happening in the flask or in the microreactor,

but the latter can markedly increase the reaction efficiency. It is also possible to use a number of parallel microreactors to perform a massive scale synthesis or screening in a given time scale.

The microreactors are also advantageous in heat transfer. Temperature is an important factor to affect the process of organic reactions. Due to large surface to volume ratio, the heat transfer coefficient of the microreactor maybe exceed several orders of magnitude than that of conventional vessel or heat exchanger.[31] The excellent heat transfer efficiency ensures the ultra-short time scale of heating and cooling in the microreactor which is very helpful for chemists to precisely control the thermal gradients in the reaction mixture. It can largely eliminate unwanted side reactions or prevent some dangerous explosion due to uneven temperature distribution in solution.

Besides the ability of precise heat control, microreactors are able to have a localized concentration control too which is almost impossible in conventional scale chemistry. In laminar flow regime, molecules can form concentration gradients as a function of positions within the channels. This advantage together with heat control makes it possible to create multiple reaction environments in a single flow-through process.

Another major advantage is that in the microreactor, it is safer to perform some very toxic or dangerous exothermic reactions than in conventional vessels. The totally enclosed environment, exact thermal and spatial control, minute reagent usage and fast reaction rate all contribute to the great safety of microreactors for organic synthesis. For example, Fortt *et al*[32] applied the continuous flow microreactor to generate diazonium reactive intermediates. The diazotisation of aromatic amines produces important industrial intermediates that are useful for the synthesis of a wide range of species including azo compounds, biphenyls, hydroxyarenes, and chloroarenes. However, the dangers of diazotisation are notoriously famous since diazonium salts are sensitive

to physical agents such as heat, light, shock, static electricity, and dehydration leading to rapid, uncontrollable decompositions and explosions. It makes the use of diazonium intermediates in industry normally subject to stringent safety procedures. Nevertheless, the author demonstrated the reactions could run very efficiently and safely in the microreactor with significant yield increase over conventional scale reactions.

Actually, there are more benefits of microreactors toward synthesis. For instance, the increased surface to volume ratio in microreactors is beneficial for surface-catalyzed heterogeneous reactions. Due to the electrophoresis effect when fluids are driven by electrokinetic pumping, it is also possible to separate the reaction products onsite to save the time and effort taken by bulk-scale reactions for post-reaction product purification. Moreover, the versatile online detection systems, such as UV-Vis, fluorescence, electrochemical detector, raman spectrometry and mass spectrometry allow direct, sensitive and real time characterization of both reactant and product information.

1.1.3 Microreactor applications

In terms of the exceptional benefits from microreactors, more and more research groups have begun to discover the potentials of microreactors in the broad fields of synthetic chemistry. Based on the fundamental studies on mass transport and fluid flow in the microreactors,[9, 33] researchers have already investigated gas phase reactions, liquid phase reactions, multi-step reactions, parallel screening of catalysts or drugs and some particular synthetic applications.

Chambers and Spink[34] first reported the use of a nickel microreactor for direct fluorination and perfluorination of organic compounds by elemental fluorine. In bulk scale, it is always troublesome for the safe handling of gaseous fluorine and exact control of temperature because

fluorination reactions are very exothermic. However, microreactors can perform this reaction easily with high efficiency. The ethyl acetoacetate can be fluorinated in 99% conversion while ethyl 2-chloroacetoacetate is in 90% conversion.

Wagner and Kohler[35] first successfully carried out continuous direct synthesis of gold nanoparticles in a microreactor. Generally, standard synthesis is difficult to control the particle size distribution that is one the most important issues in the synthesis of well-defined nanomaterials. However, the authors showed that the microreactor was able to give more uniform nanoparticles with a standard deviation of 15-30% in diameters, as a result of better control of concentration, temperature, and mass transport in the microreactor, which govern particle size distribution, i.e., nucleation and growth.

Peptide synthesis is a typical kind of multi-step reactions that has been successfully performed in the microreactors. The traditional Merrifield solid-phase-synthesis method has several disadvantages such as the need for fairly expensive polymer support and the extra steps to cleave the peptides from the support. Thus, Watts *et al*[36] have proposed a new methodology of using microreactor to synthesize peptides in liquid phase. A number of example dipeptides can be produced in significant yields in 20 min compared to 24 hr in batch reactions. The synthesis of longer chain peptides was also proved possible.

The combinatorial synthesis has been a focus of microreactor applications. The high efficiency and minute reagents usage make it superior for library synthesis than conventional methods. A simple model system for parallel synthesis is shown in Figure 1, in which two reagents in one library and two in another library would be mixed in four different combinations under pressure driven flow. Kikutani *et al*[37] presented the first example of an integrated multireactor system for 2×2 parallel synthesis on a single multilayer glass microchip. It

demonstrates the effectiveness to use continuous-flow microreactors in combinatorial chemistry. Mitchell *et al*[38] have developed a miniaturized-SYNthesis and Total Analysis System (μSYNTAS) for solution-phase compound library generation on a silicon- glass microreactor hyphenated with time-of-flight mass spectrometry (TOF-MS) for onsite compound characterization. Both serial and parallel injection methods were investigated with the sub-reactions of Ugi multicomponent reaction.

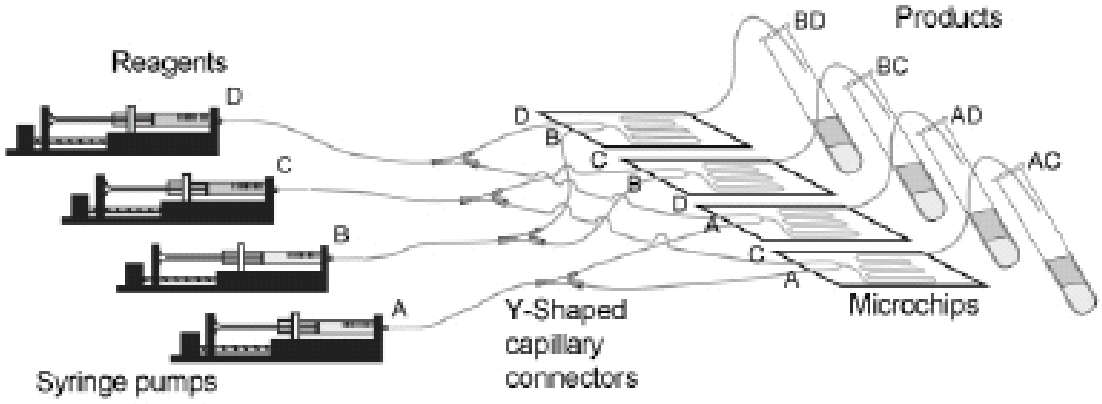


Figure 1. Schematic view of 2×2 parallel synthesis microreactor composed of four chips.

Microreactors have also been applied to catalyst discovery. Because of the benefits of microreactors for high-throughput screening, catalysts in a variety of organic reactions have been investigated. For example, Haswell *et al*[14] immobilized nickel catalysts into the capillary bed to study Kumada-Corriu reactions in a pressure-driven flow microreactor. Mikami *et al*[39] investigated the Baeyer–Villiger oxidation reactions catalyzed by fluorous lanthanide catalysts in a nanoflow microreactor system. The Baeyer–Villiger reaction is complete in a few seconds with excellent regioselectivity. Other reactions, such as enamine formation,[40] expoxidation,[41] and

multiphase reactions[42] have been performed in various microreactor systems as well, most of which are formed on chips. The typical reactions conducted in the microreactors have been summarized by Fletcher *et al*[6] that are shown in Table 1.

Table 1. Organic reactions performed in microreactors.

Reaction	*Chip material*	*Solvent*	*Conversion(%)*	*Pumping*
Suzuki	Glass	aq THF	67	EOF
Kumada coupling	Polypropylene	THF	60	Syringe pump
Aldol	Glass	THF	100	EOF
Nitration	Glass	Benzene	65	EOF
Wittig	Glass	MeOH	39-59	EOF
Enamine	Glass	MeOH	42	EOF
Ugi four component coupling	Glass	MeOH		EOF
Peptide synthesis	Glass	DMF	100	EOF
Synthesis of pyridazinones	Glass	EtOH/AcOH	30	EOF
Synthesis of amides	Glass	DCM	77	EOF
Diazo coupling	Glass	MeOH, MeCN	37,22	EOF
Aminothiazole synthesis	Glass	NMP	58-100	EOF
Knoevenagel	Glass	aq MeOH	59-68	EOF
Hantzsch thiazole synthesis	Glass	NMP	58-100	EOF
Michael addition	Glass	EtOH	95-100	EOF
S_N2 alkyl halide	Glass	DMF/H_2O	25	EOF
Dehydration	Glass/PDMS	EtOH	85-95	EOF or syringe pump
Photochemical	Silicon/quartz	2-Propanol	60	Syringe pump
Phase transfer	Glass	EtOAc	100	Syringe pump
Fluorination	Ni or Cu	Nitrogen gas	90-99	Syringe pump
Fluorination	Silicon/pyrex	MeOH	80	Syringe pump

1.2 BACKGROUND

1.2.1 Capillary-based microreactors

Currently, most published microreactor systems are formed on a chip platform. The well-developed microfabrication techniques on chip provide a high variability of reactor designs which could satisfy special reaction or operation demands. However, capillary-based reactor systems have their own merits too. They do not need complicated and costly fabrication process. Commercial capillaries are available in various diameters and materials and can be used quickly and directly by simple connection and treatment. Actually, fused- silica capillaries have long been used in capillary electrophoresis and capillary chromatography, and their properties are similar to chips in terms of high material conservation, isolation from the atmosphere, inert inner surface, and ease of transport of species by using EOF or pressure induced flow etc. Thus, under some circumstances, capillary-based microreactors can perform reactions comparable or even superior than chip-based microreactors.

Comer and Organ[43] have developed a single capillary flow system that is capable of being heated by microwave irradiation (MW). So far, almost most organic reactions conducted in chip-based microreactors are running at room temperature due to the difficulty of integration of heater components on chip. Undoubtedly, this problem limits the accessible reactions but the capillary reactor systems seem to be easier to couple heating components. This advantage may greatly improve the synthetic capability of microreactors. In their work, the reactor capillary was easy to be heated by MW and a variety of organic reactions showed excellent yield and efficiency when mixture was exposed by 4 min radiation. The more recent work by Comer and Organ[44] has also demonstrated the feasibility to develop a multi-capillary flow reactor for

parallel synthesis assisted by MW. It opens a new direction for high-throughput synthesis based on capillary instead of chip.

1.2.2 Microreactor in our laboratory

The current development of microreactor technology may seem to be promising to change the conventional batch methodology, however, a majority of researchers are still relying on the bulk reactions for synthesis and screening. The reason is that current microreactor technology is not always well-suited to the synthesis arena. The primary problem results from the absence of a kind of organic-synthesis-oriented microreactor systems. In most applications, investigators just simply borrow the setup of μTAS systems to perform organic reactions in a continuous flow stream and the small volume of chip or capillary makes this one-sample-at-a-time approach unsuitable for long time reactions. Therefore, the reactions have to be relatively fast to comply with this limitation, mostly less than tens of minutes. There are very few reports at all on flow reactors that manage slow reactions. Besides, the materials used so far to construct chip microreactors are typically glass/silicon and it is well known that glass surface is quite active and adsorbing which may act as adsorbent or catalysts for certain reactions. Moreover, the microfabrication of chip-type microreactors generally requires specialized, expensive facilities such as clean room which may be inaccessible to some organic laboratories. The last problem is that the current microreactors are still incapable of doing large numbers of combinatorial synthesis and screening. As shown in Figure 1, if the parallel synthesis is more than 2×2 scale, the whole setup will become very complicated with lots of pumps, tubes, chips or multiple channel layers if all channels are to be etched in a single chip, which demands the high integration techniques and complex instrumental operations. Therefore, in our mind, there is a

need to develop a kind of synthesis-oriented microreactor systems which should possess at least the following characteristics, e.g. sufficient chemical inertness, large capacity, high throughput, flexibility, low cost, simplicity and automaticity.

Currently, our laboratory has developed a novel microreactor system for high-throughput screening of slow reactions catalyzed by homogenous catalysts.[45] Fused silica capillary is the platform of our microreactor because of its inertness, variability, easy-heating and simple operation that are aforementioned.[43, 44] We integrate standard HPLC apparatus (pump, autosampler), syringe pumps, microinjectors, heater, fused silica capillaries and GC to build a computer-controlled reactor system in which separate zones of reactants and catalysts can be combined and loaded serially into a single reactor capillary, reacted in parallel and ejected serially for online GC analysis. Offline analysis following sample collection is also possible through the coupling of fraction collector. The design of this instrument is shown in Figure 2.

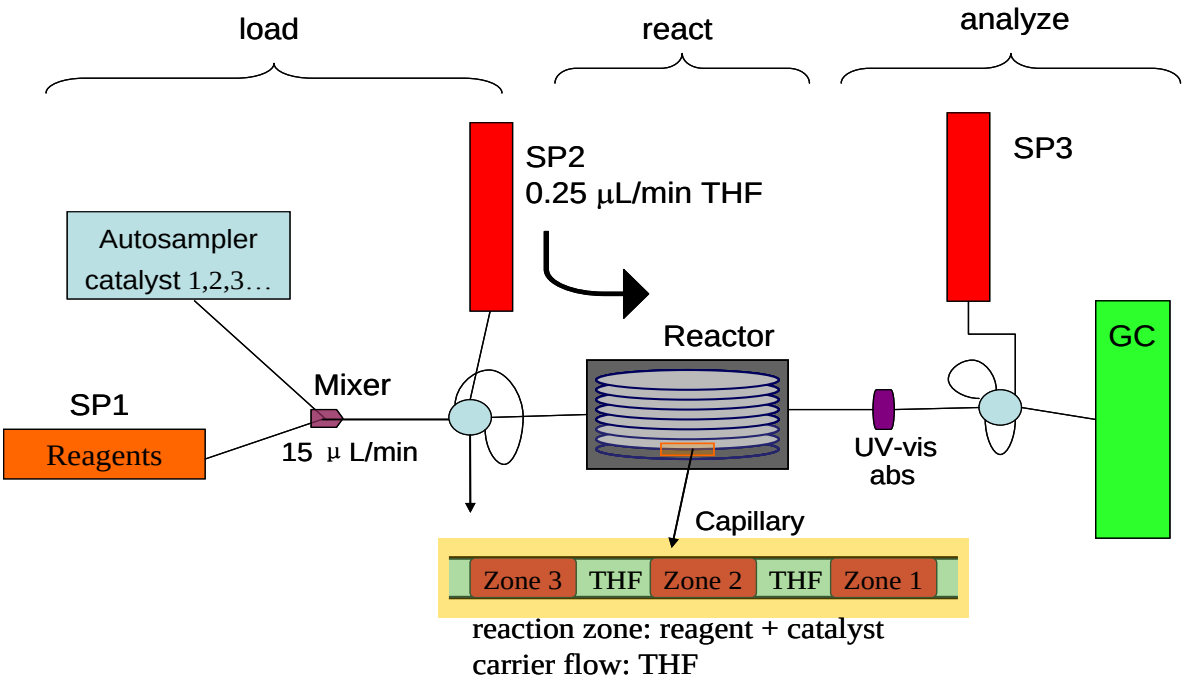


Figure 2. A schematic diagram of the microreactor system.

The loading section consists of a syringe pump (SP1) for the reagents and another for the driving flow (SP2). Catalysts are combined with reagents at equal flow rates into the loop of a 6-port microinjector by an autosampler. When the autosampler has completed its cycle, the loop's contents are pushed into the reactor. The reactor can be heated. The detection section includes a UV-Vis absorbance detector for locating the reaction zones. Under computer control, the zones are alternately pushed into one loop in the double-loop, 10-port microinjector by SP2, and then injected into the capillary GC by SP3. The injection by SP3 initiates the GC program for quantitative analysis of the reaction products.

The Y-shape mixer is formed by joining two inlet capillaries and one outlet capillary through homemade low-volume connectors.[46, 47] Catalyst and reagent are combined in the mixer and the created mixture zones will flow serially into the reactor capillary that is circled around a round flat heater coupled with temperature controller. The reactions take place in separate zones simultaneously and in parallel. Zones are defined by natural hydrodynamic dispersion with minor band broadening. Flow can be stopped for some period when reactions happen, and then zones flow out serially into the loop of microinjector which will be pumped into GC for analysis later. The instrument combines the simplicity of serial injection and detection approach used by most simple microfluidic platforms with the high throughput parallel reaction strategy which is normally far more complex than regular approach.

1.2.3 Potential applications of our microreactor

The microreactor in our laboratory is developed specifically in support of synthetic chemistry, as a result, its use should be focused on synthesis-related fields, especially in combinatorial synthesis and screening. One of the major applications would be the reaction-based catalyst

screening for the discovery of new catalysts. Generally, the discovery of organic catalysts necessitates the generation of a large library of potential catalysts followed by the high-throughput screening to find out reactive catalysts based on hundreds to thousands of reaction assays. Our microreactor will be advantageous to do this job in terms of its high efficiency and automation. Another potential application would be the discovery of new reactions. This is similar to catalyst discovery, in which combinatorial strategies would generate large libraries of transient reaction intermediates while microreactor would screen the highly active species as reagents or catalysts for the subsequent reactions.

Besides, our microreactor may also be useful for the reaction optimization or the study of reaction kinetics. A large set of data such as reaction parameters or kinetic information can be obtained through reaction assays performed in the microreactor.

The pharmaceutical screening is also likely to be placed in the microreactor. Like the catalysts, we can screen the reactivity of drugs, drug intermediates or biologically important molecules based on some physical or chemical reactions, e.g., the interactions between drug and target or enzyme and substrate etc. These interactions are in the field of molecular recognition and we believe our microreactor is possible to become a useful tool to study the recognition process too.

In sum, the purpose of our project is to develop novel methodologies in chemistry based on microreactor technology, hence, we should try to put the miniaturization concept into as many fields as possible to validate the approach of the microreactor and develop new methodologies derived from this technology which can be a significant step to revolutionize the conventional way we think and work in chemistry and even biology.

1.2.4 Stille reaction test

Shi *et al*[45] have made the preliminary optimization of the reactor system to define experimental conditions, and also had the tentative screening of catalysts for the Stille reaction to evaluate the performance of microreactor components. The Stille reaction is an important carbon-carbon bond formation reaction with metal-ligand complexes catalysts which are typical for many organic reactions. The combination of metal precatalysts and diverse ligands is able to generate a large library of potential complex catalysts from which reactive catalysts can be found by high-throughput screening methodology. In their work, a variety of palladium(II) and palladium(0) metals complexes with some common ligands were tested for catalysis of reaction between PhI and $Bu_3SnCH{=}CH_2$ to produce styrene. The screening results showed that the catalyst composed of 2 mol% $PdCl_2(CH_3CN)_2$ + 6 mol% $AsPh_3$ was the most reactive with a yield of about 65% in 5 hr's reaction. This stoichiometry and the reaction time were in agreement with traditional synthesis, proving the validity of the system. The reproducibility was also remarkable.

1.2.5 Peptide catalyzed aldol reaction

After the successful validation of screening system on known Stille reactions, we turned our attention to one of the major applied fields of our microreactor, the discovery of unknown organic catalysts. In collaboration with the UPCMLD center, we wanted to use the microreactor to screen the peptides/mimics for chiral catalysis in the direct asymmetric aldol reaction.[48, 49] The aldol reaction is believed to be one of most powerful and efficient carbon-carbon bond formation reactions. The classic aldol reaction suffers from the problems of regio- and

stereoselectivity, and the asymmetric synthesis requires the use of chiral auxiliaries and the preactivation of substrates such as converting ketone into enol or enolate first to raise its activity. However, nature uses it in different strategy. Under physiologically mild conditions, most aldolase enzymes are able to catalyze the direct aldolization of two unmodified carbonyl compounds with high efficiency and enantioselectivity, which drives scientists to investigate enzyme-like asymmetric catalysts for direct aldol reactions.[50-52]

So far, researchers have identified two types of aldolases, Class I aldolases involve enamine formation process while Class II aldolases catalyze the reaction through a Zn^{2+} cofactor. Inspired by those natural enzymes, researchers have developed two types of aldolases mimics with an emphasis on Class I type using enamine mechanism. Class I mimics generally consist of amino acid or peptide catalysts such as aldolase antibodies which could activate the donor ketone via enamine formation and the acceptor aldehyde through hydrogen bonding.[53] The Class II mimics are normally some kinds of bimetallic catalysts containing zinc or other metals complexes.[54]

Small-molecule Class I mimics like amino acids or small peptides/mimics have aroused more interests in recent years.[50, 53, 55-57] The pioneering work of List and Barbas III proved the small amino acid, L-proline, can catalyze the direct asymmetric aldol reactions in moderate or good activity and selectivity that are comparable to aldolase antibodies.[58] The catalysis of proline proceeds via an enamine mechanism[59] (Figure 3) which inspires researchers to broadly study amine-based metal-free asymmetric organocatalysts, among which small peptides/mimics catalysts are one of the research focuses now. It is supposed that the poor structural variability and small size of proline molecule are responsible for its varied reactivity towards different substrates and the low catalysis efficiency that often necessitates the use of large amounts of

catalysts. However, peptides contain more active sites with larger diversity in functional groups and structures which may result in higher reactivity and selectivity than proline or other small-molecule catalysts. Researchers believe that peptides may be a good hybrid between small rigid organocatalysts and huge enzymes.[48]

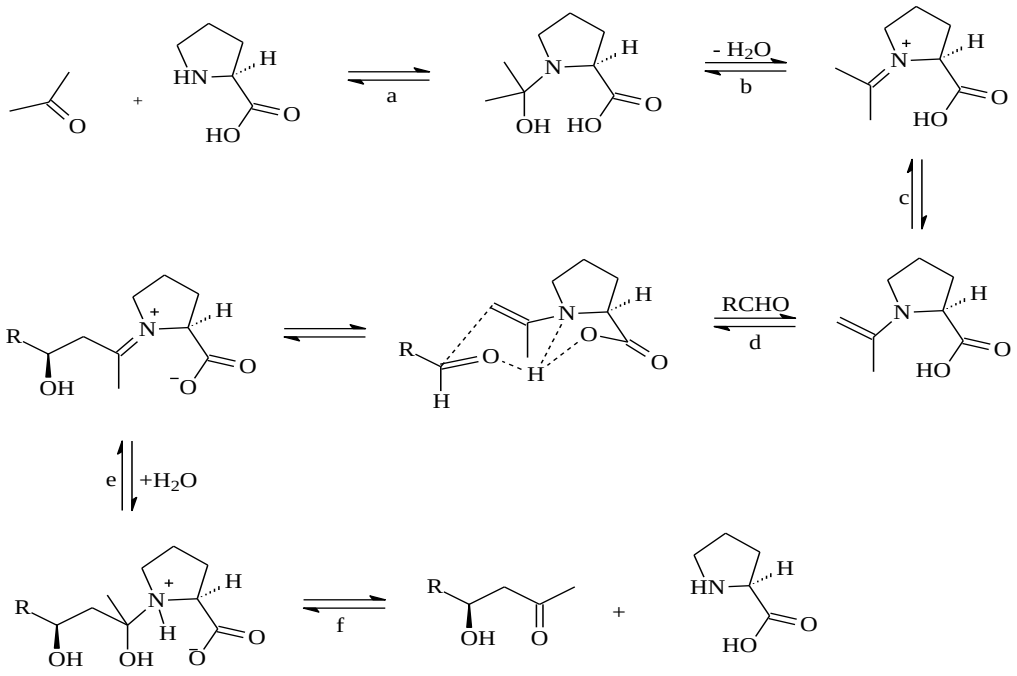


Figure 3. Proposed mechanism of the L-proline-catalyzed aldol reaction.

However, the discovery of peptide catalysts is not such a simple process that can work under rational design. Researchers generally take the combinatorial synthesis and high- throughput screening to find out reactive peptides for direct aldol reactions.[60-62] Some recent publications have described the discovery of a number of active peptides/mimics by means of conventional solid–phase combinatorial chemistry.[48, 63, 64] However, the use of microreactors to discover new peptides has not been reported yet. We believe that our microreactor has an advantage of

performing high-throughput screening which can greatly accelerate our steps toward the generation of reactive peptide catalysts for direct asymmetric aldol reactions.

1.3 STUDY PLAN

To best understand the peptide catalyzed direct asymmetric aldol reactions and fit it into our microreactor system, I plan to perform the following experiments in sequence.

1.3.1 Selection of aldol reactions

There are a large number of aldehyde and ketone candidates available for aldol reactions. The literature normally uses a two-step approach. First, select a typical aldol reaction between aldehydes and ketones for initial study of experimental conditions and preliminary screening of some supposed potential catalysts. Second, study the reactivity of good catalysts obtained from the first step by more aldol reactions in a variety of aldehyde and ketone substrates. The peptides that show good catalytic activity for most aldol reactions would be chosen to undergo further reaction optimization to achieve best yields or ee.[56, 58]

I would like to follow the above route in my experiments. At first, I will select a typical aldol reaction. The aldol reaction between nitrobenzaldehyde and acetone is widely used to study the peptidic catalysis in the literature.[51] I think it is advantageous to carry out this reaction in our microreactor because we could easily look up the reaction conditions from the literature and make a comparison of screening results. However, if this reaction is inappropriate in our system due to the instrumental limitation, I will choose another commonly studied aldol reaction for the

preliminary screening experiments. This reaction should have common reactants, simple reaction conditions and GC analyzable products.

1.3.2 Studies on reaction conditions

After the selection of a specific aldol reaction, I would perform preliminary optimization of the reaction conditions in the microreactor including solvent types, catalyst concentration, temperature and reaction time. I want to use an example peptide with enough reactivity to catalyze the selected aldol reaction and study the reaction conditions. This peptide may be selected from some known active peptides reported in the literature, or unknown peptides synthesized by ourselves through reasonable design. The widely studied L-proline, will also be regarded as a good catalyst and be used for reaction test as a comparison to peptides.

The optimization would include two steps. First, I need to select a good cosolvent for aldol reaction. Normally, acetone works as both reactant and solvent, and its large excess is important to minimize the side reactions between aldehyde and proline or other secondary-amine organocatalysts.[50] Besides acetone, in the literature there is always at least one cosolvent used in the reaction such as DMSO, THF, water, DMF to improve peptide solubility or reaction conversion. It is purported that cosolvents play a key role in peptide-catalyzed direct aldol reactions. Moreover, the selection of solvents is also extremely important for our microreactor system because the reactor can only perform homogeneous catalytic reactions and the solubility of peptides in organic solvents will be a key consideration in my work.

The second step is to study some important reaction parameters to maximize the reaction conversion or enantioselectivity. The parameters may include concentrations of reactants or catalysts, reaction temperature and time. The reaction yield or enantiomeric excess (ee) can be

analyzed by online GC coupled with achiral or chiral column for fast and accurate characterization. Through above experiments, the appropriate reaction cosolvents and best conditions will be used for the following preliminary screening of catalysts.

1.3.3 Preliminary catalyst screening

I would like to test a number of example peptide catalysts in the microreactor with the specific aldol reaction and the best reaction conditions obtained in the previous step. The peptides may be some known active peptides reported in the literature, or some unknown peptides synthesized by ourselves or purchased commercially. I would focus on peptides containing one or more proline residues which are believed to be key active sites for chiral catalysis.[48] Moreover, the selected peptides should show significant differentiation of activities and properties like solubility, polarity or molecular conformation etc., which will be good for the validation of our screening approach. The potentially good catalysts after screening would be tested with other types of aldol reactions or function as representative catalysts for further optimization of reaction conditions to get optimum yields and ee. Thereafter, a large library of peptides can then be screened based on above optimum conditions with the most appropriate type of aldol reactions.

I will show the detailed work motivated by this proposal in the next several chapters. The future work after this proposal will also be described in the last part of this paper.

2.0 SELECTION OF ALDOL REACTION

2.1 INTRODUCTION

To select a typical aldol reaction for catalyst screening is our first step. There is a variety of aldehyde and ketone substrates, in which *p*-nitrobenzaldehyde and acetone are the most commonly used reactants to test the catalysis of peptides in direct asymmetric aldol reactions. Under room or sub-zero temperature, the peptide-catalyzed aldol reaction can produce β-hydroxyketone up to 98% yield and 90% ee within 4 hr.[48]

The aldol products of *p*-nitrobenzaldehyde and acetone are generally determined by chiral phase HPLC analysis, and chiral GC is rarely used in the literature because the main product 4-Hydroxy-4-(4-nitrophenyl)-2-butanone is very non-volatile with a high boiling point of 385.6 ^{o}C. Even the byproduct from aldol condensation has a boiling point of 322.1 ^{o}C. The regular GC in our microreactor system has a maximum inlet temperature of 350 ^{o}C, and the Chiraldex GTA column we use to separate chiral enantiomers can not be run more than 180 ^{o}C. So, the retention time of aldol products in GC would be so long that fast analysis within several minutes is almost impossible. We have tried a variety of GC conditions to analyze the aldol products of *p*-nitrobenzaldehyde and acetone but the separation is hard to finish in short time which would compromise the reactor's throughput significantly.

In view that the online GC analysis is not fast enough to determine the reaction products of *p*-nitrobenzaldehyde and acetone, we have to select another typical aldol reaction to comply with the limitation of instruments. In the literature, the reaction (Figure 4) between benzaldehyde and acetone is also a kind of representative aldol reactions used to test peptides reactivity, although the yield and ee are normally lower than those of nitrobenzaldehyde and acetone. For example, List *et al*[58] performed L-proline catalyzed aldol reactions, in which benzaldehyde and acetone gave a yield of 62% and ee of 60%, while *p*-nitrobenzaldehyde and acetone gave a yield of 68% and ee of 76% under the same conditions. But it is not a big difference since benzaldehyde and *p*-nitrobenzaldehyde have very similar structures and reactivity, so I think benzaldehyde would be fine for our screening experiments.

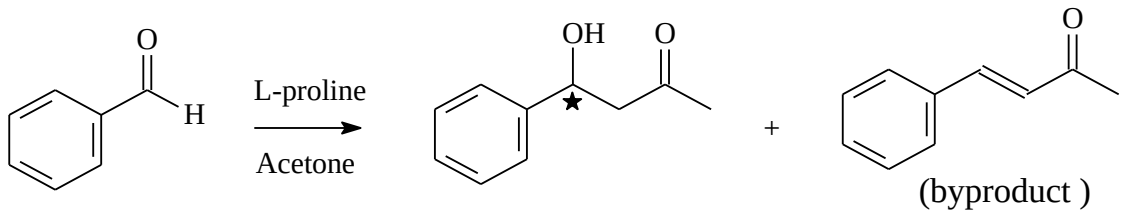


Figure 4. L-proline catalyzed aldol reaction between benzaldehyde and acetone.

The product 4-Hydroxy-4-phenyl-2-butanone has a lower boiling point of 286.4 ^{o}C, which can be analyzed by GC in limited time after optimization of conditions. The separation of enantiomers is a bit challenging because there is little literature using chiral GC column to determine aldol products with such high boiling point. The Chiraldex GTA column is good to separate oxygen-containing analytes like alcohols or ketones, and before the start of product separation, we tested the column's performance by analyzing two pairs of standard enantiomers with similar chiral functional groups. In addition to chiral column, the achiral regular column is

also used to determine reaction conversion which can be compared with results of chiral column for approach validation.

2.2 EXPERIMENTAL SECTION

2.2.1 Chemicals and materials

ACS grade THF, DMSO, acetone, methylene chloride, benzaldehyde and dodecane were purchased from Sigma (St.Louis, MO). Two pairs of enantiomers (S)-(+)-4-phenyl-2–butanol, (R)-(-)-4-phenyl-2–butanol and methyl(S)-3-hydroxy-3-phenylpropanoate, methyl(R)-3-hydroxy-3-phenylpropanoate were purchased from Fisher Scientific. (Fairlawn, NJ) L-(-)-proline was also from Fisher Scientific. Nitrogen, argon, hydrogen and compressed air were purchased from Valley National Gases Inc. (Washington, PA)

2.2.2 Microreactor Instrumentation

Syringe pumps were purchased from Harvard Apparatus Inc. (Holliston, MA). The Waters M-45 pump was from Waters Corporation (Milford, MA). The HP 1050 autosampler were purchased from Agilent (Palo Alto, CA). VICI 6-port injector (Model No: E60) and VICI 10-Port VALVE (Model No: EPCA-CE) were purchased from Valco Instruments Co, Inc (Houston, TX). The Focus GC was purchased from Thermo-Electron. It has a single column and detector (FID). The achiral column (RTX-5, 7 m × 0.32 mm (0.25 μm thick phase)) was obtained from Restek Corporation (Bellefonte, PA). The chiral column (CHIRALDEX GTA, 20 m × 0.25 mm (0.125

μm thick phase)) was obtained from Advanced Separation Technologies, Inc. (Whippany, NJ). A USB 2000 optical fiber UV-vis absorbance detector was purchased from Ocean Optics. Inc. (Dunedin, FL). A pump (Model No: CP-DSM) for injection into the GC was purchased from Intelligent Motion systems, Inc. The heater for the organic reactions was constructed locally. The temperature controller for the heater was from Minco Products Inc. (Minneapolis, MN). The fused-silica capillary with 75μm I.D., 360μm O.D. that was used as the microreactor was purchased from Polymicro Technologies, L.L.C (Phoenix, AZ).

2.2.3 Sample preparation

The standard pairs of enantiomers were prepared in methylene chloride solution with a concentration of 50 mg/mL followed by chiral GC analysis. The standard aldol condensation byproduct 4-Phenyl-3-buten-2-one was also prepared in methylene chloride at 50 mg/mL for peak characterization.

The aldol reaction was performed in the regular vial at room temperature for 24 hr with 50 μL benzaldehyde, 10 mol% L-proline and 1 mL acetone. The product mixture was analyzed by GC directly without purification.

2.2.4 GC analysis

The conditions of chiral GC for determination of standard enantiomers were that oven temperature was initially at 70^{o}C, then increased from 70^{o}C to 150 ^{o}C at 15 ^{o}C/min and held at 150 ^{o}C for 1 min. The split ratio is 50 and injection volume is 0.2 μL.

The conditions of chiral GC for determination of aldol products were that oven temperature was initially at 130^{o}C, then increased from 130^{o}C to 150 ^{o}C at 6.5 ^{o}C/min and held at 150 ^{o}C for 1 min. The split ratio is 50 and injection volume is 0.2 μL.

The conditions of achiral regular GC for determination of aldol products were that oven temperature was initially at 80^{o}C and held for 0.3 min, then increased from 80^{o}C to 200 ^{o}C at 100 ^{o}C/min and held at 200 ^{o}C for 1 min. The split ratio is 50 and injection volume is 0.2 μL.

2.2.5 Calibration curve and the yields of aldol reaction

A calibration curve was prepared from standard solution of 4-Phenyl-3-buten-2-one (aldol byproduct) and dodecane (internal standard) with varied molar ratios. The curve correlated the GC peak area ratio to the molar ratio of aldol products which can aid to calculate the reaction yield accurately.

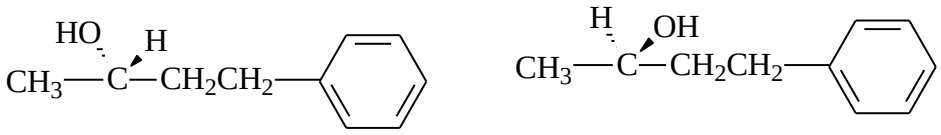
2.3 RESULTS AND DISCUSSION

2.3.1 Chiral column test

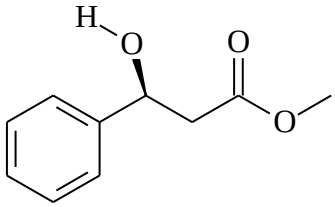
The purpose of testing chiral column with these commercial compounds is that we want to prove the column's capability of separating enantiomers with hydroxy chiral center like aldol product. And moreover, since there are no commercially available aldol product enantiomers, we have to derive the approximate separation conditions from some standard enantiomers with similar chiral centers, which is a more efficient approach than optimizing GC conditions directly from aldol

product mixture. The structures of two pairs of enantiomers are shown in Figure 5 and GC chromatograms are shown in Figure 6.

Pair 1: (S)-(+)-4-Phenyl-2-butanol (R)-(-)-4-Phenyl-2-butanol



Pair 2: Methyl (S)-3-hydroxy-3-phenylpropanoate



Methyl (R)-3-hydroxy-3-phenylpropanoate

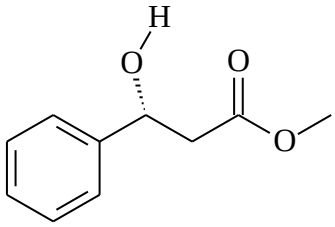


Figure 5. Structures of two pairs of enantiomers.

From the GC chromatogram shown in Figure 6, we can see that both pairs of enantiomers are well separated in short time by Chiraldex GTA column. Peaks from left to right are solvent CH_2Cl_2, (R),(S)-3-hydroxy-3-phenylpropanoate and (R),(S)-4-Phenyl-2-butanol. It demonstrates the good separation capability of GC chiral column and also provides valuable information for the further analysis of aldol products.

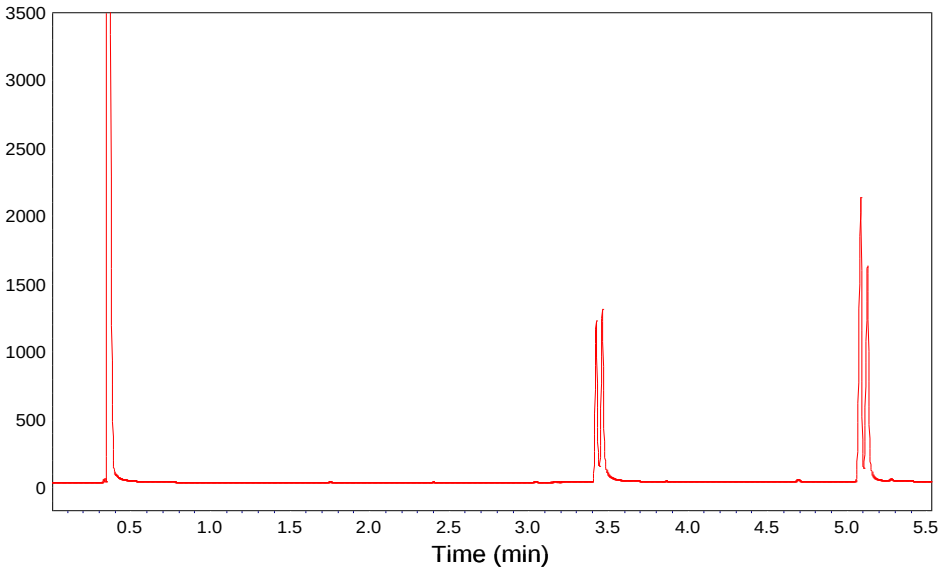


Figure 6. Chromatogram of two pairs of enantiomers by chiral separation.

2.3.2 Separation of aldol products

The aldol reaction between benzaldehyde and acetone are conducted in the regular vial at room temperature. The products are analyzed directly by GC without any pretreatment.

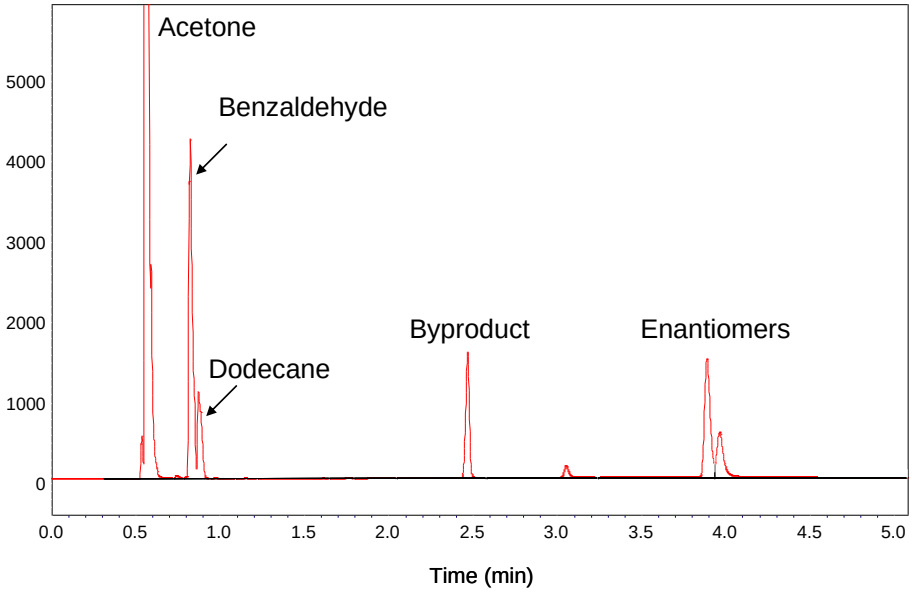


Figure 7. Chromatogram of aldol products separated by chiral column (Chiraldex GTA).

In Figure 7, chiral GC column separates aldol products in 5 min but the peaks of enantiomers have approximately 10% overlap. It is very hard to achieve absolute baseline separation because GC is still less powerful than HPLC for chiral separation which leads to most investigators prefer chiral HPLC to determine aldol products. Nevertheless, we think 10% overlap maybe create a little error on peak integration but it is all right for parallel screening of peptides because we can compare the catalysts activities based on their relative yields rather than absolute yields.

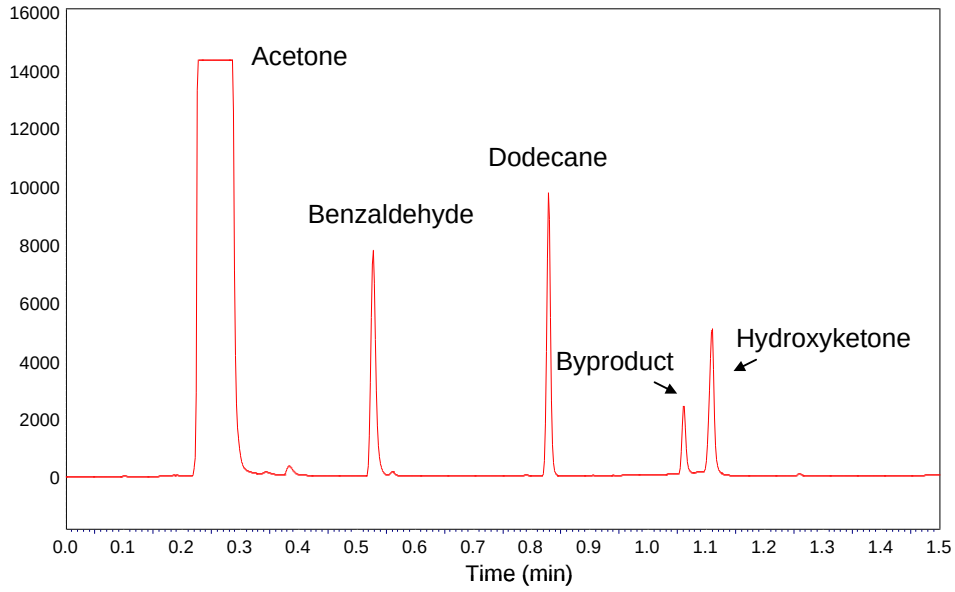


Figure 8. Chromatogram of aldol products separated by regular achiral column (RTX-5).

In order to validate the accuracy of yield determination by chiral GC analysis, I also tried the achiral regular GC to analyze the aldol products. The resulting chromatogram is shown in Figure 8, in which enantiomers form only a single peak labeled as 'Hydroxyketone'. The yield calculation by means of molar ratio of β-hydroxyketone to dodecane shows that chiral column always gives a bit smaller ratio of β-hydroxyketone to dodecane than achiral column that means the calculated yield by chiral analysis would be smaller than true value. The reason is still

unknown, however, this problem can be solved by making two calculation curve for chiral and achiral analysis respectively.

In fact, the problem of chiral column is not only the yield determination. The Chiraldex GTA column is very vulnerable to water or moisture, and even minute amount of water present in the sample may hydrolyze the stationary phase leading to the loss of chiral selectivity. However, it is hard to create a water-free environment in the microreactor to perform the aldol reaction. Therefore, we want to have a flexible use of both columns for analysis of aldol products. For example, we can study the reaction yield mainly by achiral column first and determine the enantioselectivity by chiral column. This approach can largely reduce the chance of chiral column to contact water-containing samples which is good to protect the expensive chiral column.

2.4 CONCLUSION

In this section, I chose the aldol reaction between benzaldehyde and acetone as the model reaction for the next screening experiments. Both chiral and achiral columns of GC have been tested with aldol products and separation conditions have been optimized to fit for the requirements of online analysis in the microreactor system. As a result of the vulnerability of chiral column to water or moisture, we would like to use both columns flexibly to determine the aldol products. The achiral column is mainly for reaction conversion while chiral column is primarily for ee determination. This combination can greatly protect the chiral column and minimize the error from chiral analysis.

3.0 OPTIMIZATION OF ALDOL REACTION

3.1 INTRODUCTION

Generally, the optimization of organic reactions should consider the following parameters: reactant concentration, types of catalyst and concentration, solvent, temperature, time or stirring speed etc. In our experiment, we need to find an optimum reaction condition that is applicable for most aldol reactions in the microreactor. Consequently, we have to consider not only the generality of reaction conditions for different peptides catalysts, but also the feasibility to make the aldol reactions appropriate for the microreactor.

The first consideration of us is the selection of good solvents. The routine way in the literature is to add acetone in excess amount as both the reactant and solvent which can minimize the side reaction between proline and aldehyde. However, besides acetone, researchers always use other cosolvents in the reaction too to enhance the reaction conversion or improve the catalyst's solubility. These cosolvents normally include DMSO, DMF, THF, NMM, water or methanol.[50, 57, 63] DMSO is supposed to be one of the most suitable cosolvents for peptide-catalyzed aldol reaction because its strong polarity is able to assist in the formation and stabilization of separated charges of enamine that is the rate-limiting step in the aldol reaction.[65] Compared to other cosolvents, DMSO can always give better reaction conversion and enantioselectivity in shorter time.[56]

However, one of our concerns is the solubility of catalysts. It is reported that amino acid L-proline is only slightly soluble in DMSO, needless to say other larger peptides that may be harder to be soluble in pure DMSO, and acetone is perhaps even worse than DMSO. The regular reactor can make the catalysts dissolve gradually in the solvent as reaction goes on, but to our microreactor, it is necessary to prepare totally soluble catalyst solution before the start of reaction. Therefore, it will be a focus of our work to find out more appropriate cosolvents to solve the difficulty of peptides solubility.

After studying the cosolvents, we may go on to study other reaction parameters such as temperature, catalyst concentration or reaction time to optimize the reaction yield and ee. In order to make the reaction run smoothly in the reactor, we probably need to make some changes of the current components of the microreactor to fit for the special demand of aldol reaction. L-proline will be used as one of the typical catalysts for reaction optimization because it is easily obtained and has been widely studied to be a moderate catalyst for regular aldol reactions. Moreover, it has similar properties to many hydrophilic peptides such as solubility and polarity which can represent peptide catalysts to some extent. Nevertheless, we still need to find an active peptide to replace L-proline for the optimization process. The peptide may be more representative than L-proline for peptide-catalyzed aldol reaction.

3.2 EXPERIMENTAL SECTION

3.2.1 Chemicals and materials

ACS grade DMF, DMSO, acetonitrile, pyridine, N-methyl morpholine and acetic acid were purchased from Fisher Scientific (Fairlawn, NJ). Dioctyl Sulfosuccinate (Aerosol OT) was also from Fisher Scientific. Others are same to chapter 2.

3.2.2 Optimization experiment

The optimization of reaction parameters was primarily carried out in the microreactor. A certain amount of L-proline or other peptide catalysts was dissolved in 1 mL cosolvents such as DMSO or THF etc. The catalyst vials were placed on the tray of autosampler which would push the catalyst samples into the loop of a microinjection at a flow rate of 15 μL/min. The reactant benzaldehyde (50 μL) was mixed in acetone (3 mL) containing 30 μL dodecane as the GC internal standard. The reactant solution was pumped by a syringe pump and combined with the catalyst at an equal flow rate before entering the loop (0.75 μL) of microinjector. Thus, the loop contained both reactant and catalyst. The loop contents were injected into the capillary reactor to form one reaction zone via THF that is pumped by another syringe pump at a flow rate of 0.25 μL/min. The reactor consists of 75 μm-i.d., 6.7-m-long piece of fused-silica tubing and up to 20 reaction zones can be loaded serially in the reactor. The flow was stopped after all the samples were loaded into the reactor and aldol reaction was carried out for varied time at room temperature. After reaction, THF syringe pump was turned on at an equal flow rate of 0.25 μL/min and zones were pumped out of reactor serially and flowed into the loops (0.75 μL) of 10-

port microinjector. The contents of the loop were pushed into the GC with M6 pump triggered by the adsorbance detector. The GC run time is equal or less than the sample loading/injection times.

3.2.3 Statistical calculation

All the reactions were carried out four times in the microreactor. The yield and ee were calculated by calculation curve and results are shown in standard deviation.

3.3 RESULTS AND DISCUSSION

3.3.1 Solvent selection

The L-proline or most hydrophilic peptides are hardly soluble in the pure organic solvent, such as acetone, THF, methanol, DMF or even DMSO. The poor solubility is a major problem for us since we have to keep the catalyst concentration to a certain level that may catalyze the reaction to produce significant yield that can be analyzed and differentiated by GC. However, this difficulty is rarely reported in the literature which primarily utilizes traditional approach to perform the reaction. Thus, we have to figure out some ways to improve the solubility of catalysts by ourselves.

We first try to add a kind of surfactant named dioctyl sulfosuccinate (Aerosol OT) into the THF cosolvent to help dissolve L-proline. The solubility of L-proline in THF with Aerosol OT is very good, but the reaction yields are much lower than L-proline in DMSO only, although the

latter cosolvent contains fewer L-proline catalysts. The comparison is shown in Figure 9, in which a series of L-proline concentrations are studied to elucidate the difference.

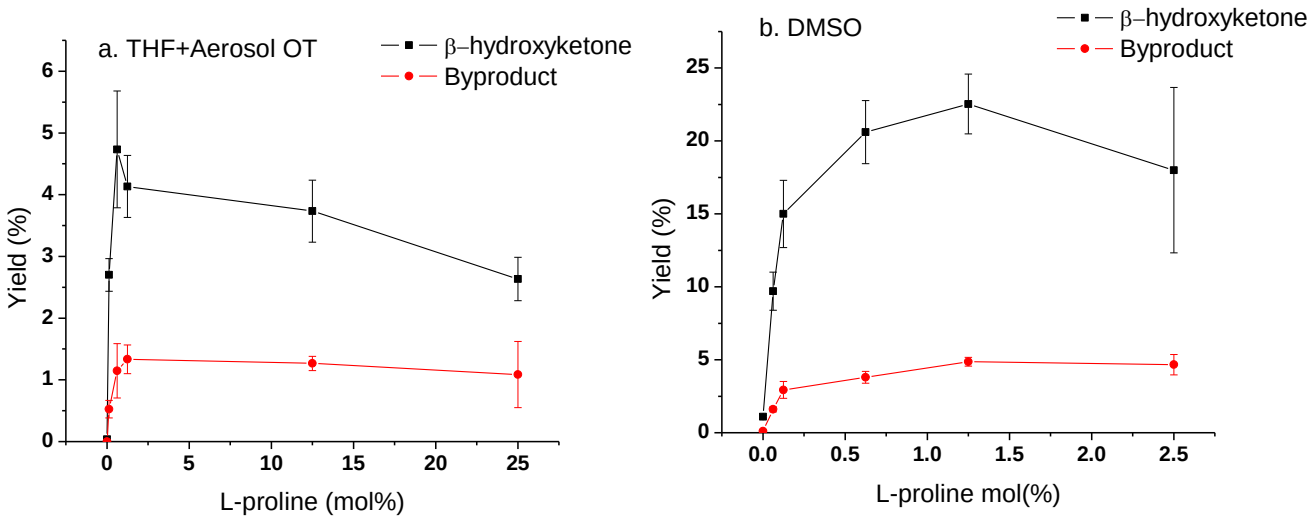


Figure 9. The yield of L-proline catalyzed aldol reaction with different solvents. The composition of reaction zone: 0.2 mol/L benzaldehyde, DMSO or THF and acetone is 1:1 (v/v). Reactions were done in the microreactor at room temperature for 24 hr, and results are shown in standard deviation (n=4).

From the Figure 9, we can see the reaction yields in both THF+Aerosol OT and DMSO show the similar tendency with increasing L-proline concentrations. Owing to the low solubility of L-proline in DMSO, 2.5 mol% is almost the maximum concentration whereas L-proline in surfactant solution is possible to have much higher concentration. However, higher concentration seems to be of little help to increase conversion, but actually causes worse conversion. The reason is because too much L-proline can lead to more side reactions such as oligomerization reaction. For example, some trimer (BAA, one benzaldehyde and two acetone molecules), tetramer (BAAA) or pentamer (BAAAA) products may be produced from the aldol addition of

several benzaldehyde and acetone molecules, which would reduce the yields of dimer products that we expect. This problem will be further studied in the later parts of this report with the proofs obtained from more experiments.

In addition to the poor yield of reaction performed in THF+Aerosol OT, a serious problem caused by surfactant is that the proline-surfactant solution may precipitate out some cotton-like precipitation gradually when catalyst solutions sit for some time. This precipitation will clog the valves and capillaries severely and the cleaning is very time- consuming. It is not clear what the precipitate is, but there are some strange phenomena. For example, if I add some acetone manually in the proline-THF-Aerosol OT solution, the precipitation would form a bit faster. The THF solution containing high concentration L-proline and high concentration surfactant forms precipitation faster than the solution with low catalyst and surfactant concentrations. Based on above observations, I hypothesize that it is perhaps due to the instability of L-proline-surfactant micelles in pure organic solvent. The catalyst-surfactant solution may be in a metastable state at first as a result of fast ultrasound dissolution and then within some time, the micelles may gradually look for new equilibrium state which can then lead to some precipitation. This process could be accelerated by some parameters such as changes of temperature, concentration, or solvents etc.

The poor yield in THF+Aerosol OT may also result from the formation of micelles that perhaps affect the chiral catalysis of L-proline by changing its conformation or blocking its functional groups. What's more, the surfactant also increases the viscosity of the solution which can result in the change of flow dynamics in the microreactor too. Thus, the use of surfactant is not a good way to improve peptide solubility. We have to find other methods that are good for both catalysis and aldol reactions.

During the study of surfactant-catalyst method, we found that precipitation is a really weak point of our microreactor system. Any small precipitates may cause serious clogging in the tubing and valve. Actually, this problem has already been found when Stille reaction was tested. Because it only happened occasionally, so it had not been solved yet. However, I think it is necessary to add some protecting parts in the reactor system to prevent any unexpected precipitation and make sure the reactor can work smoothly. Hence, I added three HPLC nano-volume filters into the sample loading part, one is in the autosampler tubing, the other two are in the tubing of syringe pump I (reactants) and syringe pump II (THF) (Figure 10). These filters are effective to stop any solids, impurities or precipitation entering the reactor tubing and valves. It seems a good improvement of the reactor system.

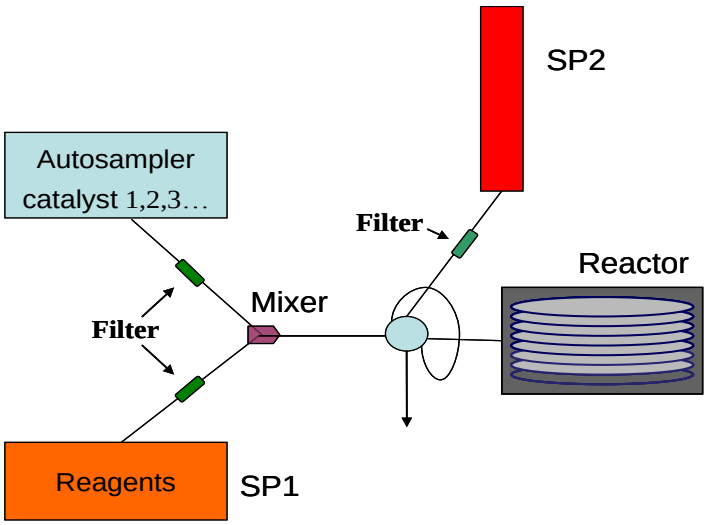


Figure 10. The schematic diagram of filter-included loading part of the microreactor.

After the failure of surfactant approach, I tried another method by adding acetic acid into the cosolvent to make catalysts soluble. Acetic acid is often used to dissolve sparingly soluble hydrophilic peptides in the organic solvents. The weak acidity has little effect on the structure

and conformation of soluble peptides. Depending on the concentrations of acetic acid in solution, varied concentrations of catalysts can be obtained, even for some extremely insoluble peptides. Therefore, acetic acid may be a good supplementary chemical to improve catalyst solubility.

I compared the aldol reaction yields in a variety of common solvents containing a small amount of acetic acid. The reactions were catalyzed by L-proline. The experiments were carried out in the regular vials instead of the microreactor because I only want to make sure if DMSO or other literature reported solvents are suitable for aldol reaction and look at if there is any precipitation formed during reaction when I use the acetic acid. Thus, it is not necessary to perform the reaction in the microreactor and the conventional approach is fine to test cosolvents. The results are shown in Figure 11.

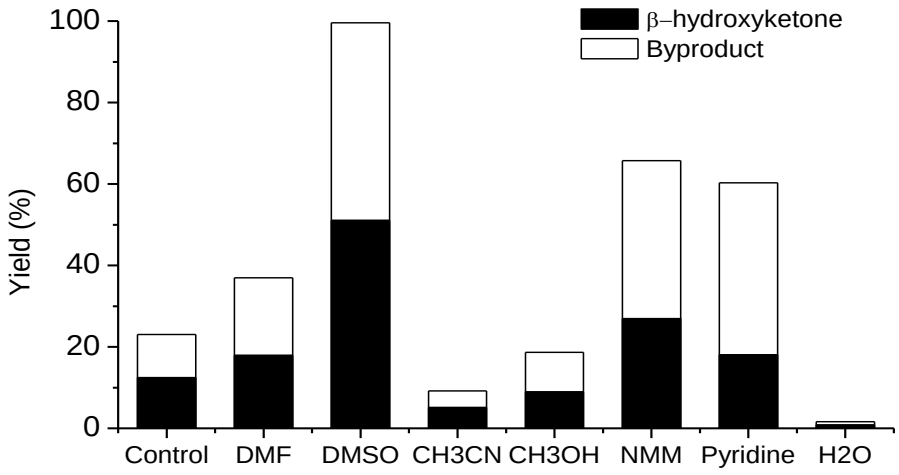


Figure 11. The testing of cosolvents for aldol reaction.

Reactants: 20 μL benzaldehyde + 100 μL acetone. Catalysts: 2 mol% L-proline in 25 μL acetic acid. Cosolvents: 50 μL. The reactions were done in vials for 24 h at room temperature. The yield was analyzed by GC. The control reaction had no cosolvents.

From Figure 11, it is clear that DMSO is the best one which gives highest yield for both β-hydroxyketone and byproduct. The β-hydroxyketone yield in DMSO is about 53% which is close to the literature reported 60%.[58] The aldol reaction without any cosolvent shows much lower yields. It is in agreement with the literature. Besides DMSO, N-methyl morpholine (NMM) also shows significant conversion, which has also been reported in a few papers that NMM perhaps functions like DMSO to increase aldol conversion. Therefore, I think it may be meaningful to have a detailed study of this less used solvent in our microreactor to see if it can be a good substitute of commonly used DMSO.

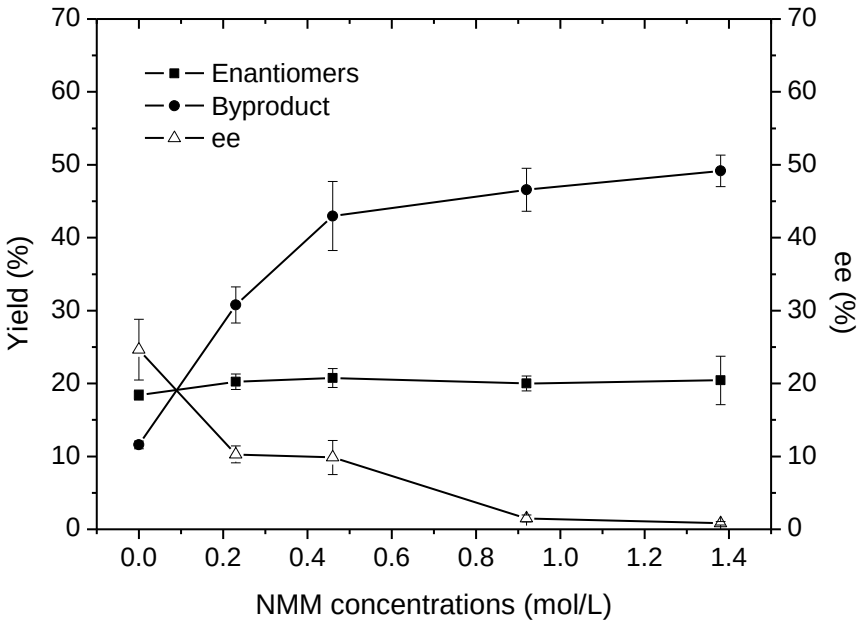


Figure 12. The effect of NMM concentrations on the proline catalyzed aldol reactions. The reaction zone: 0.05 mol/L benzaldehyde, 10 mol% proline, 0.9 mol/L acetic acid in acetone. The reactions were done in microreactor for 24 h at room temperature and ee was analyzed by chiral GC. Results are shown in standard deviation (n=4).

The effect of NMM concentrations on reaction yield and ee is shown in Figure 12. From above figure, we can see that the increasing NMM concentrations seem to be good only for byproduct and there is little increase in the enantiomers' yield. The enantioselectivity becomes actually worse with more NMM in solution, which manifests that NMM is not good for aldol reaction. These results contradict a few papers, and I think it is probably due to the different reaction environment or reaction conditions, e.g., microreactor versus bulk reaction, and more acetic acid may also weaken the basicity of NMM to change its effect on reaction.

Anyway, NMM is not an appropriate cosolvent in our system. Thus, like most researchers, I would select DMSO as the cosolvent to dissolve catalysts because it has been widely proved to be a good solvent for aldol reaction.

3.3.2 Optimization of reaction parameters

In the literature, peptide-catalyzed aldol reactions are often carried out at room temperature or 0 ^{o}C for 4-24 hr with catalyst concentrations ranging in 10-40 mol%. I will study these parameters in the microreactor to obtain the optimum conditions.

Figure 13 shows the effect of catalyst's concentrations on the reaction yield and ee. Normally, the reaction yields should increase in proportion to the catalyst's concentrations to a certain level until reaches maximum conversion and then the conversion would become independent of catalyst's concentrations. However, in our experiment, higher L-proline concentrations lead to lower enantiomers' yields which are similar to the results obtained in Figure 9. But the byproduct's yields are rising almost linearly with increasing catalyst's concentrations.

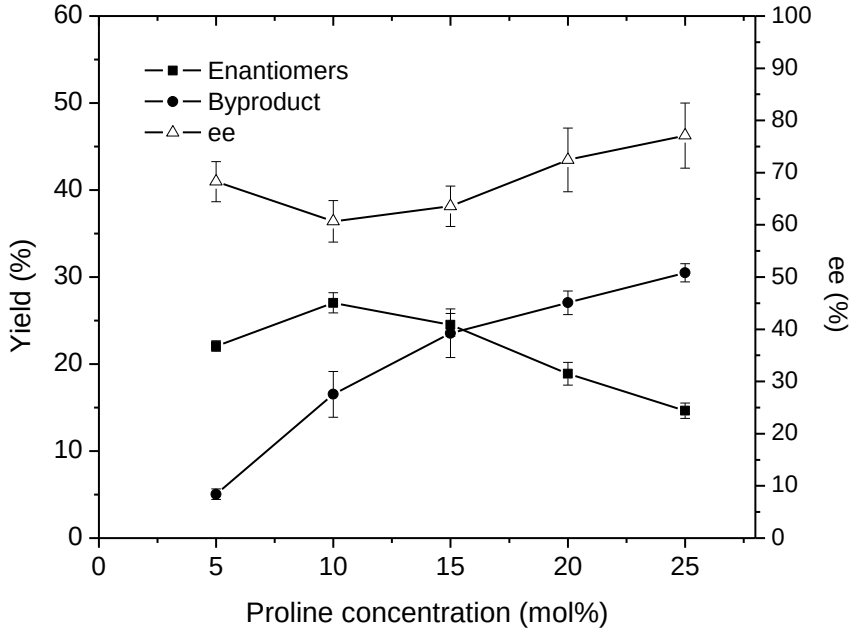


Figure 13. The effect of L-proline concentrations on the yield and ee of aldol reactions. The reaction zone: 0.05 mol/L benzaldehyde, 0.9 mol/L acetic acid, DMSO and acetone in 1:1 (v/v). The reactions were done in microreactor for 24 h at room temperature and ee was analyzed by chiral GC. Results are shown in standard deviation (n=4).

The reason has been described at earlier text, that is, too much proline would cause more oligomerization side reactions, such as forming trimer (BAA), tetramer (BAAA), or higher oligomers via aldol addition or condensation. These reactions would consume the dimer products of single aldehyde and acetone and reduce their conversion markedly. The hypothesis has been proved by GC-MS analysis which has identified the significant presence of the trimer (BAA) produced by aldol condensation of one benzaldehyde and two acetone molecules. However, the byproduct from aldol condensation of benzaldehyde and acetone is increasing almost linearly, and it is probably because the hydroxyketone is much easier to catalyze by L-proline for further

aldol addition or condensation than α,β-unsaturated ketone. There is less consumption of the byproduct which then shows expected rise of yields with increasing catalyst's concentrations. The little increasing ee is probably because catalyst may have a preference to catalyze one enantiomer over the other to form large oligomers.

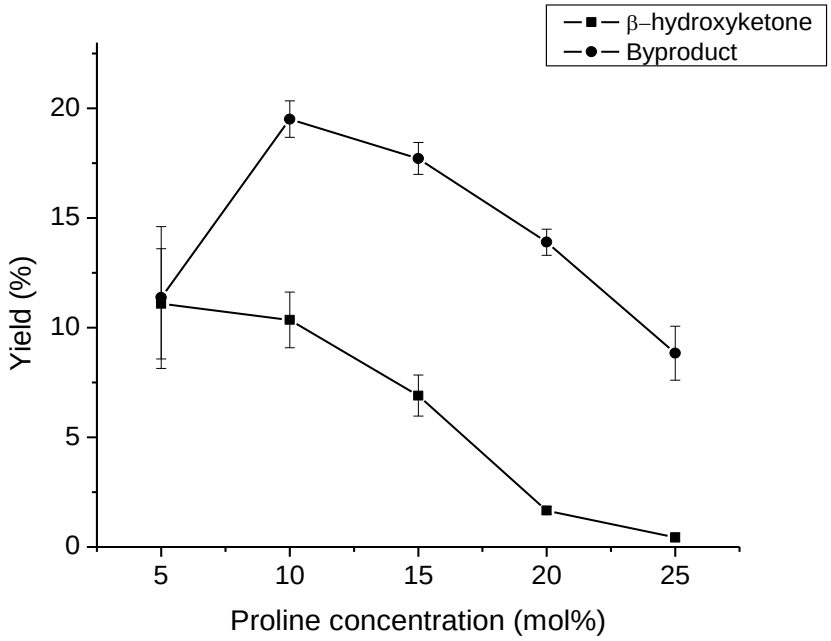


Figure 14. The effect of proline concentrations at higher temperature.

The composition of reaction zone: 0.05 mol/L benzaldehyde, 0.9 mol/L acetic acid, DMSO and acetone in 1:1 (v/v). The reactions were done in the microreactor for 24 h at 50 ^{o}C. Results are shown in standard deviation (n=4).

Normally, the temperature has a positive effect on reaction yield because it can accelerate the reaction rate markedly. However, in our experiment, the temperature is in adverse effect. In Figure 14, it is obvious that 50 ^{o}C is high enough to cause larger yield loss to both aldol product and byproduct. The byproduct's yields show the similar decreasing tendency to those of

enantiomers which means at this temperature, the L-proline has been active enough to catalyze the byproduct into oligomers too. The higher the catalyst's concentrations, the lower the reaction yields. Thus, to keep the temperature low is necessary to minimize side reactions, which has been a common protocol in the literature by carrying out reactions in room temperature or zero degrees Celsius. We would perform the reaction at room temperature because it is much easier to carry out in the microreactor.

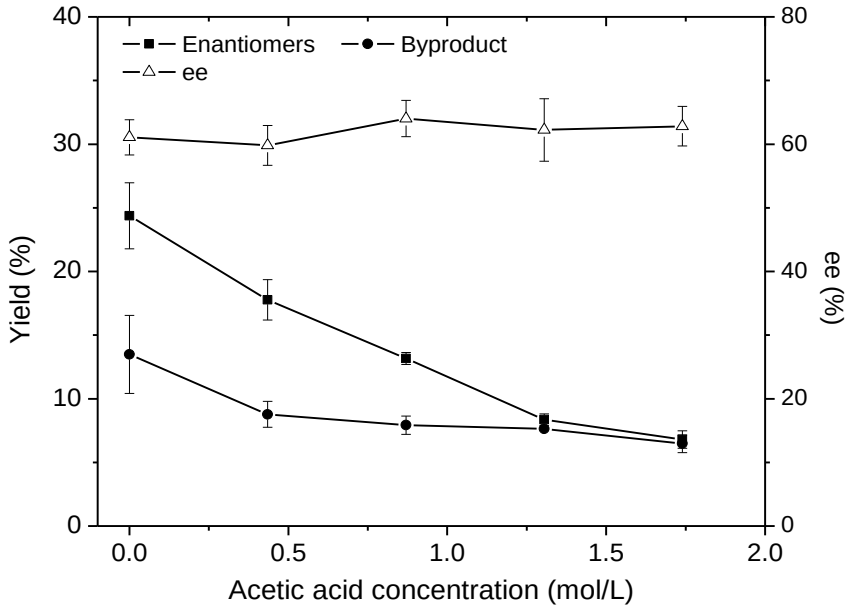


Figure 15. The effect of acetic acid concentrations on the reaction yield and ee. The composition of reaction zone: 0.05 mol/L benzaldehyde, 10 mol% proline, DMSO and acetone in 1:1 (v/v). The reactions were done in the microreactor for 24 h at room temperature. Results are shown in standard deviation (n=4).

The concentration of acetic acid is another important parameter. In Figure 15, it shows that acetic acid has an adverse effect on reaction yields. Both enantiomers and byproduct show reducing conversions. However, the enantioselectivity seems to be little affected by acetic acid.

Theoretically, acids maybe function as a double-edged sword toward peptide-catalyzed aldol reaction. On the one hand, acids can catalyze the process and increase the conversion by protonation of the carbonyl group to enhance its electrophilicity, while on the other hand, acids can also protonate the secondary amine of proline to lower its nucleophilicity which would slow down the enamine formation and reduce the conversion.[66] The effect mainly depends on the type and acidity of acids. However, the enantioselectivity should be little affected because acids do not change the conformation of catalysts that is believed to be the key factor for catalysis.

Our experimental results prove that acetic acid has a negative effect on the reaction conversion but little influence on ee. Nevertheless, the use of acetic acid is still feasible in our experiments if we can control the level of acetic acid in solution to make the side effect minimal. As long as the reaction can give significant yield for comparison and differentiation, the parallel screening should be all right because I think every catalyst would have comparable yield loss and unchanged enantioselectivity. It is a simple and effective method to solve the solubility problem.

The last important parameter is the reaction time. According to literature, the aldol reactions catalyzed by peptides have varied reaction times ranging from several hours to several days in order to obtain best conversion, which primarily depend on the types of reactants, catalysts, solvents, temperature and so on. However, for our project, screening efficiency is very important to work on large amounts of peptide candidates, so I think it is a good approach to select an optimum reaction time from a very reactive peptide that represents a standard time for all candidates. So far, since L-proline is the most active catalyst, I would study its reaction kinetics to find the optimum time.

The results are shown in Figure 16 in which reaction times are ranging from 1 hr to 24 hr. It seems that the reaction has reached maximum yield at 4 hr, and further increasing time has little

effect on the yields of both enantiomers and byproduct. 4 hr is a relatively short time and can be taken as a standard reaction time for peptide screening.

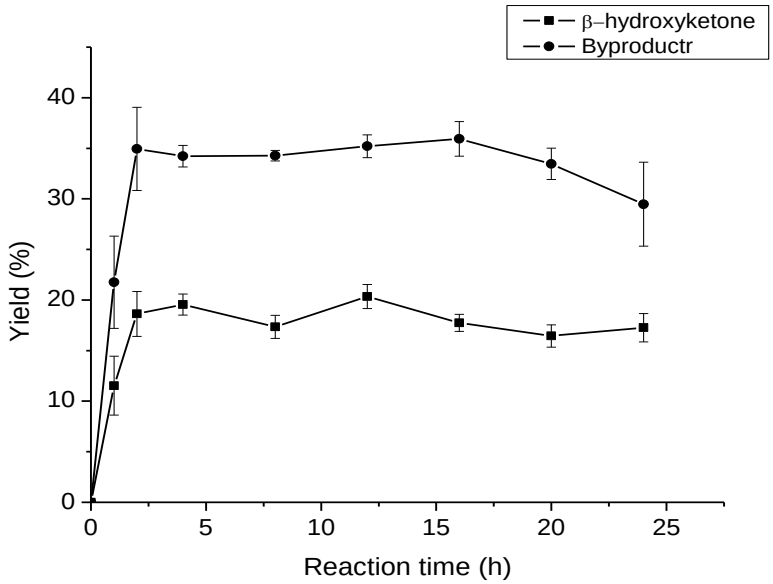


Figure 16. The reaction kinetics of proline catalyzed aldol reaction.

The composition of reaction zone: 0.05 mol/L benzaldehyde, 10 mol% proline, 0.9 mol/L acetic acid, DMSO and acetone in 1:1 (v/v). The reactions were done in the microreactor at room temperature. Results are shown in standard deviation (n=4).

3.4 CONCLUSIONS

In this section, I prove that DMSO is the best solvent for peptide-catalyzed aldol reaction which is in agreement with literature. A supplementary chemical acetic acid is used to improve peptide solubility to make the reaction fit for our microreactor. The effect of catalyst concentration is studied and a moderate concentration of L-proline catalyst can give best yield and ee as a result

of the serious side reactions induced by high concentration L-proline. The reaction is good to be conducted at low temperature and reaction time is about 4 hr to achieve maximum yields. The acetic acid does have a little negative effect on reaction conversion, but reaction ee is of little influence. Therefore, we would use a small amount of acetic acid in our experiment to minimize its side effect.

4.0 PRELIMINARY SCREENING OF PEPTIDES

4.1 INTRODUCTION

In the previous part, I study the reaction parameters in the microreactor with a typical catalyst L-proline. Strictly speaking, L-proline is not a peptide and its properties must have some difference with peptides. Therefore, I think it is important to discover one or more active peptides to take place of L-proline for further reaction optimization. The discovery of these peptides can be based on the screening of some commercially available peptides or rational designed synthetic peptides. The reaction would be performed in the microreactor and conditions are borrowed from L-proline catalyzed aldol reaction. After the screening, active peptides would be used for further optimization of reaction parameters which can then be regarded as a general procedure for the following screening of large libraries of peptides.

The selection of representative peptides is based on the idea that proline-containing peptides are more likely to be reactive than none-proline peptides. From the studies in the literature, the peptide that has a N-terminal proline residue and a free carboxylic group in proximity to the secondary amine maybe have higher catalytic activity than other normal peptides.[48, 63, 67] Thus, we would also try to screen some peptides containing N-terminal proline residue at first. It can be easy for us to compare our results with the literature, and we are also more likely to obtain some active peptides with less effort.

4.2 EXPERIMENTAL SECTION

4.2.1 Chemicals and materials

Peptides: H-Pro-Gly-OH and H-Pro-Phe-OH were purchased from Bachem (San Carlos, CA). Pro-Gly-Gly and Gly-Gly-Pro were purchased from Sigma (St.Louis, MO). Pro-Asn and Pro-Val-Asp were synthesized locally. Others are same to those used in chapter 2 and 3.

4.2.2 Peptides screening

The screening experiments were carried out in the microreactor. 10 mol% peptides were dissolved in 1 mL DMSO containing 50 μL acetic acid. The catalyst solutions were pumped into the system by autosampler. Other operations were same to chapter 3.

4.3 RESULTS AND DISCUSSION

4.3.1 Preliminary screening of peptides

The peptides used for screening experiments are as follows: Pro-Gly and Pro-Phe are reported in the literature to be reactive catalysts for aldol reaction between nitrobenzaldhyde and acetone. Pro-Asn and Pro-Val-Asp are synthesized by the UPCMLD center. Pro-Gly-Gly and Gly-Gly-Pro are available in our laboratory with unknown activities and to be screened as novel peptides. None of them have been studied to catalyze the aldol reaction in the microreactor. We would

screen their activities based on reaction yields and L-proline is used as a control to compare with peptides.

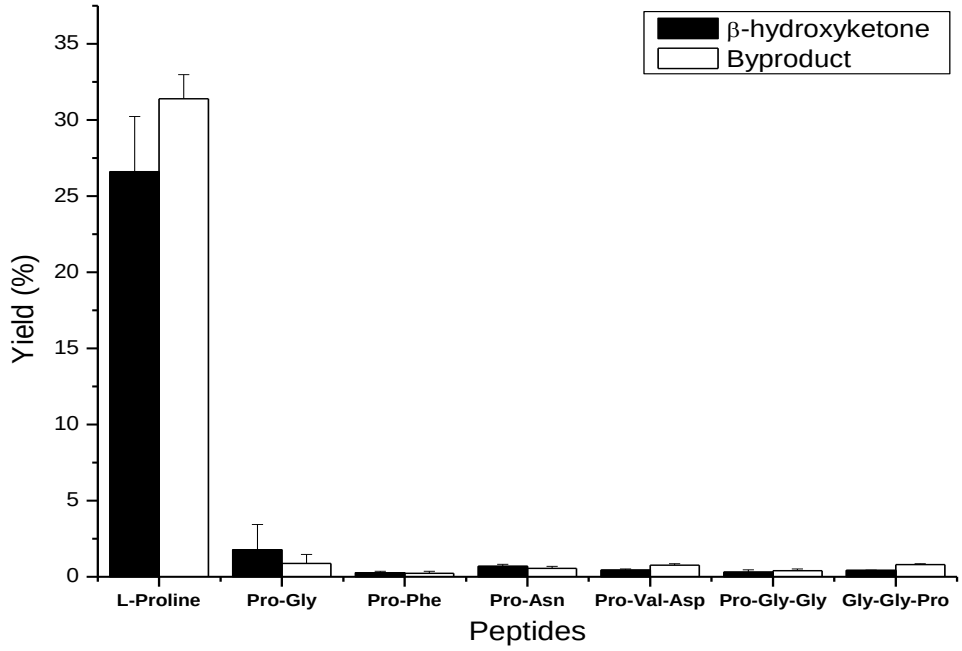


Figure 17. The screening of peptides.

The composition of reaction zone: 0.05 mol/L benzaldehyde, 10 mol% catalysts, 0.9 mol/L acetic acid, DMSO and acetone in 1:1 (v/v). The reactions were done in the microreactor at room temperature. Results are shown in standard deviation (n=4).

The results of reaction yields are shown in Figure 17. None of peptides are active enough to produce significant yields like L-proline. Though the reaction conditions may be not optimal for peptides because the conditions are obtained from L-proline, the yields should not have such a lot loss. Therefore, I think there are other reasons why these peptides are inactive. Pro-Gly and Pro-Phe are reported in two papers[56, 67] to be excellent catalysts for nitrobenzaldehyde and acetone (yields more than 95%), and they also give moderate yields for benzaldehyde and

acetone. However, in our experiments, they only give poor yields. The reason is probably due to the different reaction conditions, for example, we use acetic acid but they don't, we use 10 mol% catalysts but they use 20 mol%–40 mol% catalysts, we only use DMSO, but they use DMSO and NMM both. However, it is still not clear if these parameters can really generate such a great difference in catalytic activity.

The peptide Pro-Val-Asp has been proved to less active by the literature.[48] Pro-Gly-Gly is unlikely to be active either because from molecular modeling (Cache 6.1), it is almost a linear molecule. The β-turn in large peptide molecule is supposed to be an important factor to bring N-terminal secondary amine approximate to carboxylic group which can facilitate the process of hydrogen bond formation. Gly-Gly-Pro is not active because of the absence of N-terminal secondary amine. Thus, our experiments also prove that the position of secondary amine relative to carboxylic group is crucial to the catalysis process. However, there are also many other properties of catalysts that can influence the reaction process, such as hydrophobicity, polarity, and molecular size that may induce steric hindrance. Thus, it is difficult to estimate the catalyst activity based on simple deduction or molecular modeling.

The best way to discover a good peptide is to rationally design, precisely synthesize and thoroughly test the catalysts on a large number of reactions. So, we do need some peptides like L-proline that have been well studied by organic researchers which are more likely to be suitable for our microreactor experiments than those rarely studied peptides like Pro-Gly or Pro-Phe. I think it would be better if we can obtain a good peptide as the model catalyst for the reaction optimization. But if it is not possible to obtain active peptides from literature, we can still carry out the screening of unknown peptides with L-proline-based conditions until an active peptide is found in our own experiment.

4.4 CONCLUSION

In this section, I have a preliminary screening of several dipeptides and tripeptides. However, none of them show significant yields like L-proline. The reason is not clear, but it may be related to the different conditions between our experiment and the literature. So far, L-proline is the best catalyst from our screening which somewhat agrees with some literature that few of known peptides or aldolase antibodies are superior to L-proline in the catalysis of aldol reactions.[53] However, we would try to discover more active peptides than L-proline under the powerful support of our microreactor in the future work.

5.0 FUTURE WORK

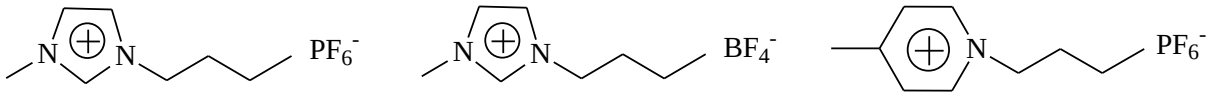
5.1 THE ALDOL CHEMISTRY

5.1.1 Peptide screening

The search for active peptides is still an important part of my future work. So far, L-proline has been tested to be the best catalyst but no active peptides have been found yet. We still need to do more screening work in search of active peptides that can work well in our microreactor. The library of potential peptides will be mainly obtained from two routes, one is to purchase some literature reported active peptides that are commercially available, and the other is to collaborate with organic group to locally synthesize some potentially good peptides or reported peptides that are commercially unavailable. Because the peptides research is still just emerging now, then it may be more realistic to have some locally synthesized peptides screened for discovery of active peptides. This approach also costs less than large purchase of peptides for screening. The preliminary screening conditions are based on those from L-proline until comparable or more active peptides are discovered which can used as model catalysts for further reaction optimization. One of the goals of our project is to apply our microreactor system to discover new peptide catalysts for direct aldol reaction that can serve both organic synthesis and combinatorial chemistry. Thus, it is very significant for us to keep searching active peptides in the future.

5.1.2 Ionic liquids as solvent for aldol reaction

In recent years, there has been growing interests in the use of ionic liquids as solvents for organic synthesis because of their special properties as reaction media.[68-71] The term of "ionic liquid" refers to liquids composed entirely of ions that are fluid around of below 100 ^{o}C. Room temperature ionic liquids are most commonly employed for organic reactions which typically consist of nitrogen-containing organic cations and inorganic anions. The normal organic cations are N-alkylpyridinium and 1-alkyl-3-methylimidazolium while inorganic anions are Cl^-, NO_3^-, PF_6^- and BF_4^- etc. The combination of cations and anions can lead to a large number of ionic liquids which provide great flexibility for chemists to select the most suitable pair for a specific reaction.[72, 73] Three examples of ionic liquids are shown in Figure 18.



(A) (B) (C)

Figure 18. Structures of two typical ionic liquids.

(A) 1-Butyl-3-methylimidazolium hexafluorophosphate (Bmim PF_6), (B) 1-Butyl-3-methylimidazolium tetrafluoroborate (Bmim BF_4), (C) 1-Butyl-4-methylpyridinium hexafluorophosphate (4mbp PF_6).

The ionic liquids have several characteristics compared with regular organic solvents. They are nonvolatile, highly thermostable and lots of organic and inorganic compounds are probably

well soluble in them. These properties are useful in the catalyst mediated organic process that needs the easy recovery of catalysts after reaction.

The employment of ionic liquids in the proline-catalyzed direct aldol reaction has already been published in some publications recently.[74-76] Most researchers obtained comparable or higher yields and ee in the ionic liquids compared with routine solvents such as DMSO. And the recovery of proline is simpler and more efficient. The recovered proline shows almost unchanged reactivity for the next catalysis that can keep for several recovery generations.

The advantage of ionic liquids in our project is that we can substitute ionic liquids for acetic acid to dissolve peptides that may prevent the side effect of acid on reaction yields. Actually, we got much lower yields than those reported in the literature which indicates that some parameters of our system may be not so good. Acetic acid is a possible factor and the use of ionic acids may be able to increase the yields to the normal level. We can combine the ionic liquids with regular solvents such as DMSO to create an optimal media for aldol reaction. The recovery of L-proline is not a concern of us although it is a major merit of ionic liquids for organic chemists.

We would study the effect of ionic liquids on aldol reaction based on the procedure used to study acetic acid. L-proline would be the typical catalyst like most literature. Reaction yields and ee would be tested by GC. If ionic liquids can give positive results, more work should be done such as reaction optimization and peptides screening.

Several important questions will also be answered in my future research of ionic liquids:

1. Could most peptides be easily dissolved in ionic liquids?
2. Are ionic liquids appropriate in our microreactor system, which means, do they lead to the occurrence of precipitation or capillary clogging? Do they have any effect on flow dynamics in the capillary due to their charges and viscosity?

3. Is there any effect of ionic liquids on GC system or product analysis because of their highly nonvolatile property?

5.2 IMPROVEMENT OF REACTOR'S FLEXIBILITY

The current capability of our microreactor is to perform one-step, homogeneous, liquid based organic reactions with GC analyzable products. In order to make the instruments capable of carrying out more types of organic reactions, some components of the system may be changed or improved as follows.

The GC is a key part of our microreactor. However, the limitation of GC mainly results from the incapability of GC to analyze high boiling point, nonvolatile reactants and products that greatly lowers the reaction variability in our microreactor system. A solution is to replace GC by HPLC that normally is more powerful than GC to analyze nonvolatile organic compounds. The specific advantages of HPLC to the aldol reaction are that we can select more reactants such as nitrobenzaldehyde and carry out more types of aldol reactions in the microreactor that are good for reaction optimization. And more, the side reactions such as aldol condensation may happen seriously during GC analysis due to high temperature employed which can then be minimized by room temperature HPLC. Thus, detected reaction yields may be higher with HPLC than GC analysis.

The connection and automated computer control have been proved possible by our laboratory. We believe the optional usage of GC or HPLC to be suitable for different reactions can greatly increase the potential of our microreactor system in support of a variety of synthetic fields.

The multi-step reactions can also be carried out in our system by adding more sample loading parts. The current system has only one set of sample loading section and it is likely to connect more loading parts to the system as shown in Figure 19.

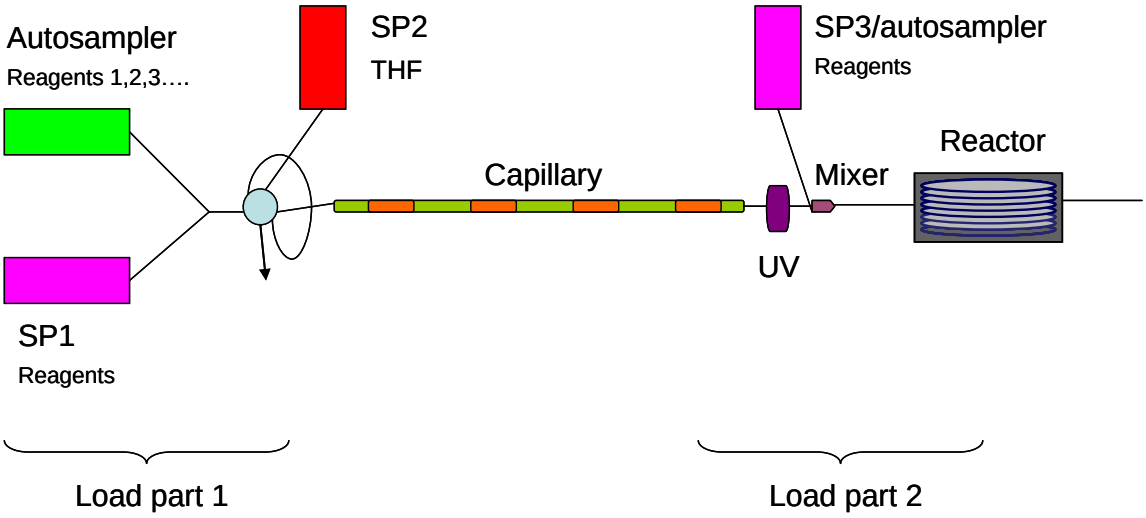


Figure 19. The schematic design of microreactor for two-step reaction.

The autosampler and syringe pump 1 (SP1) inject a series of reagent 1 and single reagent 2 into a certain length capillary for the first step reaction. When the sample zones pass through the UV detector, signals would trigger another autosampler or programmable syringe pump to inject reagent 3 to combine with the zones and then the second step reaction can happen in the reactor capillary. The other parts such as 10-port micoinjector, GC are unchanged. This design is still employing the simple approach of serial-injection and parallel reaction in a single capillary, but can contain more steps of reaction.

In addition to multi-step reaction, this design is also able to integrate the combinatorial synthesis and high-throughput screening into a whole system. For example, the discovery of new

metal-ligands complex catalysts generally requires the generation of intermediate catalysts from the combination of a library of metal precatalysts and a library of ligands followed by reaction-based screening for discovery of reactive catalysts. This whole process is possible to be carried out in our system by combining the metals and ligands in the load part 1 and conducting the screening reaction in the load part 2 after mixing the reagents into the catalyst zones.

Technically, it is possible to couple more than two syringe pumps or autosamplers to the main capillary for performing multi-step or complicated reactions. However, the precise control and synchronization may be a potential problem for more complex design. Therefore, two-step reaction is most likely to be carried out in our microreactor.

5.3 REACTOR OPTIMIZATION

The optimization of reactor's throughput has not been done yet. The current throughput is based on the preliminary optimization through the studies of Stille reaction in which maximum 20 reaction zones can be loaded serially into 6.7 m capillary reactor in 2 hr. For instance, if the system has 2 hr loading time, 5 hr reaction time and 2 hr analysis time, the throughput of the system would be 2.2 zones per hour. Thus, the purpose of the optimization is to increase the throughput of the reactor to an optimum level. The throughput normally depends on a lot of parameters, such as capillary length, capillary diameter, carrier flow rate, loop size (zone size), sample loading time, online analysis time, and reaction time etc. These parameters are normally intercorrelated and an abundant amount of work may be needed for experiments-based optimization process. Therefore, we want to use Mathcad software to have a mathematically approximate optimization first followed by actual experiments to perfect the optimized

throughput. The mathematic optimization would employ known capillary flow equations such as diffusion equation to derive the function of reactor's throughput versus a numbers of parameters mentioned above. Actually, this approach has been widely applied in many engineering fields and Mathcad is believed to be a simple but powerful tool for function-based optimization. The approximate results would be a good reference of our optimizing experiments.

In sum, I plan to perform the above several aspects of researches in the future after my comprehensive exam. The collaboration with organic groups and other lab colleagues may be necessary to have a more efficient and productive research in our project.

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