Biocatalytic micromixer coated with enzyme-MOF thin film for CO₂ conversion to formic acid

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In this study, a novel micromixer with a 3D helical, threaded channel was fabricated via 3D printing. The micromixer can enhance the mass transfer of reactants and product in an enzymatic cascade reaction converting CO₂ to formic acid. Two enzymes, including carbonic anhydrase (CA) and formate dehydrogenase (FDH), were biomineralised in a zeolitic imidazolate framework-8 composite thin film on the micromixer channel that has been modified with polydopamine/polyethyleneimine. The biocatalytic performance of the micromixer was evaluated by testing at various liquid flow rates, and an optimum liquid flow rate at 1 mL/min (Reₗ = 8, Deₗ = 3) was observed as the two-phase flow pattern in the micromixer channel transitioned from slug flow to bubbly flow. A comparison of the micromixer performance with and without threaded channels revealed ~ 170% enhancement in formic acid yield, indicating improved mixing with the presence of threads. In addition, the formic acid production rate for the micromixer with threaded channel was three folds higher than a conventional bubble column, demonstrating the superior performance of the proposed micromixer. The ease of assembling multiple micromixer units in series also enabled the immobilisation of different enzymes in separate units to carry out sequential reactions in a modular system. As a proof of concept, the solution product collected from long term biocatalysis was also tested in a direct formic acid fuel cell, which showed a promising prospect of integrating these two systems for a closed-loop energy generation system.

1. Introduction

Micromixer technologies have attracted significant research interests in the chemical and biological fields for applications such as chemical synthesis, RNA/DNA analysis, PCR amplification, and detection of chemical contents [1–3]. In particular, passive micromixers are simple to operate as enhanced mixing occurs following the change in structure along the channel length, allowing easy integration with other devices [1,4]. Various structures or features in the microchannel can be implemented to optimise the mixing efficiency, including Y or T shaped multi-input channels [5–7], interdigital channels [8–10], zig-zag channels [11], channels with staggered herringbone grooves [12], network of interconnecting channels defined by glass fibre strands [13], and also a combination of the different mixing units [14]. Furthermore, the introduction of a gas phase into the micromixer in a segmented gas–liquid flow has also been shown to enhance advection or mixing in the liquid [15]. Many researchers have started to report on the use of micromixers for gas–liquid dispersions as gas–liquid contacting is involved in many industrially relevant processes. The large gas–liquid interfacial areas due to the small dimensions of the microchannels can significantly improve the gas–liquid mass transfer rate, which is well suited for applications such as gas absorption. Yue et al. [16] and Hou et al. [17] investigated the mass transfer characteristics of micromixers with rectangular channel and arborescence channel respectively through the absorption of CO₂ gas into water and alkaline solutions. The volumetric mass transfer coefficients of both mixers were found to be at

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least one or two orders of magnitude higher than that of traditional contactors such as bubble column and packed column [16,17]. Given the enhanced mass transfer performance of micromixers compared to traditional contactors, opportunities exist to implement micromixers for biocatalytic reactions involving a CO$_2$ gas phase and a liquid phase. An example applied in this study is the synthesis of formic acid via enzymatic cascade reaction of CO$_2$, CO$_2$ gas, along with a solvent, can be introduced into a micromixer where CO$_2$ is first hydrated to bicarbonate in a reaction catalysed by carbonic anhydrase (CA) [18,19]. This is then followed by a reduction reaction to formic acid catalysed by NADH-dependent formate dehydrogenase (FDH), which requires a redox cofactor (NADH) as the terminal electron donor [19–24]. In this reaction, the reduction of bicarbonate to formic acid is accompanied by the oxidation of NADH to NAD$^+$. Changes to the microenvironment of an enzyme, such as pH and temperature, outside its optimal range can lead to a loss of bioactivity and even denaturation [25]. Therefore, enzyme immobilisation on a solid support is a popular strategy to enhance the stability of enzymes in operational and storage conditions [26–28]. Furthermore, enzyme immobilisation allows facile separation from the product, which improves the recyclability of enzymes [29]. In addition, immobilisation of multiple enzymes at adjacent positions (spatial immobilisation) also reduces the diffusion distance of intermediate product to the subsequent enzyme in the cascade reaction, which can improve the product yield [19]. Metal-organic frameworks (MOFs) have gained increasing interest over recent years as a platform for enzyme immobilisation. They are constructed from metal ions and multidentate ligands, which self-assemble through coordination bonds into 3D extended structures. A diverse range of MOFs has been synthesised and explored over the past two decades with exceptional properties such as high specific surface area, well-defined pores, and outstanding thermal and chemical stability, making them popular candidates for enzyme immobilisation [30]. Recently, Kang et al. [28] proposed an approach of immobilising proteins, enzymes, and DNA in-situ during the crystallisation process of MOFs, which encapsulates the biomacromolecules and also serves as an effective protective coating against harsh thermal and chemical treatments. This approach, known as biominalerisation, has since then been employed in several studies with different MOFs such as zeolitic imidazolate framework-8 (ZIF-8) [31,32], ZIF-90 [33], HKUST [34,35], MIL-53 [36,37], UiO-66-NH$_2$ [38] and NKMOF [39]. Among the numerous subclasses of MOF, ZIF-8 is the most popular MOF material used for in-situ encapsulation of enzymes due to its mild and biocompatible synthesis conditions [28]. This material has also been used in a broad range of applications, including gas separation [40,41], catalysis [31,42], biosensing [43], and adsorption of organic compounds [44]. It is constructed via the self-assembly of tetrahedrally-coordinated Zn$^{2+}$ ions and 2-methylimidazolozole linkers, forming a highly crystalline microporous material with high thermal, chemical, and mechanical stability [45]. The imidazole group in ZIF-8 can act as a nucleophilic agent and react with CO$_2$ under an aqueous condition to form HCO$_3^-$ [46]. Therefore, there is a synergistic effect of ZIF-8 and CA enzyme in the hydration reaction of CO$_2$ to bicarbonate. Zhang et al. [47] and Ren et al. [48] reported higher apparent catalytic activity of up to 22-folds for carbonic anhydrase encapsulated in ZIFs compared to their free counterpart. Furthermore, the activity of the FDH enzyme in the cascade reaction of CO$_2$ to formic acid can also be improved due to the higher concentration of HCO$_3^-$ substrate produced [38]. Recently, Mukherjee et al. [49] found the formate dehydrogenase (FDH)-enzyme in-situ film synthesised via biominalerisation, the enzymes are not fully encapsulated in ZIF-8 but are instead dispersed in the ZIF-8 matrix close to the surface. This is supported by the observation that a chemical inhibitor (chymotrypsin) which is larger than the ZIF-8 pore window can access and deactivate the enzymes [43]. ZIFs can also trap enzymes in the crystal defects that occur during the biominalerisation of MOF, which provides alternative passageways for the movement of substrates larger than the ZIF-8 pore window to and from the enzymes [33,49–51]. This would allow for the movement of enzyme cofactor (NADH) to the FDH enzyme in the reduction reaction of bicarbonate to formic acid [38]. Although formic acid can be an intermediate product for subsequent enzymatic reactions to produce methanol as a liquid fuel, studies have shown that a bottleneck appears in the next reaction step catalysed by formaldehyde dehydrogenase due to the slow accumulation of formic acid [52,53]. Therefore, the question arises whether the formic acid product from biocatalysis can be utilised for other applications. Formic acid itself is a chemical of commercial interest as it can be used for tanning leather [54] and as a silage preservative [55]. Over the past two decades, several studies have also been conducted on direct formic acid fuel cells (DFAC) utilising formic acid as a fuel. DFAC exhibits several advantages over direct methanol fuel cell, such as small fuel crossover flux and high theoretical open circuit potential of 1.40 V compared to 1.21 V for direct methanol fuel cell [56]. Typically, formic acid oxidation on Pt-based catalysts proceeds through two pathway mechanisms. The desirable pathway is the dehydrogenation reaction of formic acid directly to CO$_2$, while the undesirable pathway is the dehydration reaction of formic acid that forms CO as a reaction intermediate [57]. The in-situ generated CO intermediate can readily adsorb onto the Pt surface and induce catalytic deactivation of the electrode, which impedes further fuel oxidation [58]. Wang et al. [59] found that the oxidative removal of adsorbed CO on Pt is initiated at 0.3 V for a Pt/C rotating disk electrode, due to the generation of adsorbed OH species originating from water dissociation. Therefore, it is undesirable to operate below this potential as the dehydration step acted solely as a site-blocking poison rather than a reaction intermediate. In this paper, we demonstrate the use of a novel 3D printed micromixer with unique features (threaded channel) to enhance the mass transfer in the enzymatic cascade reaction of CO$_2$ to formic acid. Two enzymes, carbonic anhydrase and formate dehydrogenase, were co-immobilised via biominalerisation in a ZIF-8 thin film on the surface of the micromixer channel. The performance of the micromixer in terms of formic acid production rate was evaluated in comparison to a micromixer without threaded channel and a conventional bubble column as a benchmark. Furthermore, the effect of liquid flow rate as well as spatial (domino) immobilisation of enzymes on formic acid yield were also investigated. Lastly, as a proof of concept, the biocatalysis product collected from the micromixer was tested in a direct formic acid fuel cell to demonstrate that it is a viable fuel source for the fuel cell using commercial Pt/C catalyst, indicating the potential integration of these two catalytic systems for portable, green energy generation.
study were fabricated using a SLA 3D printer (Form2, Formlabs, USA) in Clear Resin V4 (RS-F2-GPCL-04). The parts were first drafted in SolidWorks 2018 x64 (Dassault Systèmes SolidWorks Corporation, Waltham, MA, USA), a commercially available CAD software, and then exported as .stl file, which is a proper file format for 3D printer software packages. Our channel has two inlets, one for buffer with NADH solution and one for CO2 gas, as well as one outlet. The file is then imported into the Preform software for pre-processing of the printing procedure. 50 µm layer thickness was selected to make sure that the parts have enough quality and accuracy while auto support generation was used to create supports for proper attachment of the part to the build plate. When the printing process is completed, the parts were removed from the build plate carefully and washed three times with IPA thoroughly. The internal holes of the channels are 2.3 mm in diameter, which is proper for removing uncured resins. The threads in the channel are 0.77 mm in width and 0.35 mm in height. Lastly, the parts were post-cured in a UV-curing chamber.

2.5. Enzyme labelling for fluorescence microscopy

The procedure for enzyme labelling used was based on our previous study with some modifications [43]. 4.5 mg of FITC was dissolved in 0.5 mL of DMSO, which was subsequently mixed with 7 mg of CA in 2 mL of sodium carbonate-bicarbonate buffer (50 mM, pH 9.2). In a separate vial, 50 µL of 2 mg/mL atto 550 NHS ester was added slowly into 7 mg of FDH prepared in 2 mL of sodium carbonate-bicarbonate buffer (50 mM, pH 9.2). Following this, the FITC-CA and atto 550-FDH mixtures were shaken at 300 rpm in darkness for 2 h. Subsequently, the unreacted enzymes were separated from the labelled enzymes by using Illumra NAP-25 column (GE Healthcare Life Sciences) eluted with Milli-Q water. The first band eluted contains the dye-labelled enzyme, which was collected and used for in-situ immobilisation in ZIF-8 thin film. Fluorescence microscopy imaging was conducted using IX53 Inverted Microscope from Olympus at 490 nm for the FITC labelled enzyme and 554 nm for the atto 550 labelled enzyme.

2.6. Enzyme activity assay and precipitation test of hydrated CO2 to CaCO3

20 mL Tris-HCl buffer (0.05 M, pH 7) containing 1 mM NADH was recirculated in the micromixer at different liquid flow rates of 0.5–3 mL/min. In the case of a long term experiment conducted where the product of biocatalysis was to be utilised in a formic acid fuel cell, 50 mL of Tris-HCl buffer containing 15 mM NADH was used instead. The experiments were all conducted in batch mode. CO2 gas was introduced into the micromixer channel via another inlet at a flow rate of 0.3 mL/min controlled by a metering bellows sealed valve (Swagelok), which was connected to a buffer tank. A schematic drawing of the experimental setup is provided in Fig. 1A. For the bubble column, the ZIF-8/CA&FDH in-situ particles were added into 20 mL of buffer solution containing NADH in a sealed bottle, and CO2 gas was bubbled at a flow rate of 0.3 mL/min from the bottom using a hypodermic needle (B Braun TM). The length-to-diameter ratio of the bottle is 2. This value usually varies between 2 and 5 for bubble columns used in biochemical applications [63]. UV–Vis spectrophotometer (Cary 300, Agilent Technologies, Australia) was used to measure the concentration of NADH periodically in the reaction solution at a wavelength of 340 nm. The formic acid yield was calculated based on the amount of NADH consumed, as described in the literature [20].

CaCO3 precipitation test was carried out to determine the amount of hydrated CO2 present in the buffer solution, based on the procedure given by Hou et al. [18]. 0.2 g of CaCl2 was dissolved in 5 mL of 0.5 M Tris-HCl buffer (pH 9.6), which was then mixed with the 20 mL Tris-HCl buffer solution containing hydrated CO2. The mixture was left to precipitate for 5 min before filtering with a filter paper. The amount of CaCO3 precipitated was measured by weighing.

3. Results and discussion

3.1. Principles of mixing

CO2 gas and buffer solution containing the enzyme cofactor (NADH) were introduced into a micromixer via two separate inlets (Fig. 1A). The CA and FDH enzymes immobilised in-situ in ZIF-8 thin film on the micromixer channel reduce CO2 to formic acid via a cascade reaction. This involves an initial hydration step of CO2 to bicarbonate catalysed by CA enzyme, which is a zinc-based metalloenzyme. At the active site of CA, a zinc-bound hydroxide attacks CO2 carbon to form metal-bound bicarbonate, which is then displaced by an external water molecule [64,65]. This is followed by a reduction reaction of bicarbonate to formic acid catalysed by NADH-dependent FDH in an aqueous environment [19,38]. The bicarbonate substrate and NADH bind to the active center of FDH comprised of amino acid residues, where NADH serves as the terminal electron donor in the redox reaction [66]. The formation of 1
mol of formic acid requires 1 mol of NADH [19–24].

In order to increase the mixing index (MI) and volume of sample processed, a 3D structure was employed for the micromixer geometry. The third dimension of the micromixer structure allows the system to rapidly achieve and maintain a high MI over the length of the channel at both high and low Reynolds numbers [67]. In addition, the presence of threads in the micromixer channel increases the area of contact between the gas and liquid, which can enhance the mixing efficiency especially at low Reynolds number where diffusion is the dominant mixing mechanism. Hence, this device overcomes a common problem faced in many planar micromixers where they suffer from low mixing efficiency at the diffusion mixing regime. The designed threads around the channel also prevents particle settling by making the fluid flow from one side of the channel to the other. Furthermore, another benefit of this device over other typical micromixers is the uninterrupted presence of chaotic advection generated by Dean flow in the curved channels [68]. In contrast, the Dean flow is limited to a certain length and area due to the fabrication limitation of a planar micromixer. As the micromixer could achieve ~ 100% mixing efficiency across different Re at the early loops (Fig. 1C), the system is well suited for applications requiring controlled reaction of samples.

3.2. Characterisation of micromixer surface

SEM and AFM analysis were conducted to examine the surface morphology and roughness of the pristine, PDA/PEI modified, and ZIF-8/CA & FDH in-situ thin film on the micromixer surface (Fig. 2). The surface of the pristine micromixer is shown in Fig. 2A, which has a root mean square (RMS) surface roughness of 11.3 nm (Fig. 2B). The addition of dopamine hydrochloride and PEI formed a uniform layer of PDA/PEI on the micromixer surface due to a reaction between the amine group in PEI and catechol group in PDA (Fig. 2C), which can proceed through Michael addition or Schiff base reaction [69]. The surface roughness remained comparable to that of the pristine surface (Fig. 2D). Subsequently, the chelation of Zn metal ion by the catechol group in PDA enables the nucleation and growth of a highly intergrown thin film layer of ZIF-8 (Fig. 2E). There was an increase in surface roughness to 42.5 nm due to the presence of ZIF-8 microstructures (Fig. 2F). The micro surface roughness of ZIF-8 is not expected to have a significant effect on the bulk flow behaviour and mass transfer as the relative roughness (surface

Fig. 1. A) Schematic illustration of biocatalytic micromixer setup used for the reduction of CO$_2$ to formic acid, with an enlarged view showing the 3D structure of the micromixer channel with threads to promote mixing. The intricate structures and features of the micromixer have been fabricated using 3D printing method. The product from biocatalysis was then utilised in a formic acid fuel cell for energy generation. B) The arrangement of two micromixers in series with CA and FDH enzymes immobilised in separate micromixers (domino immobilisation) for the enzymatic cascade reduction of CO$_2$. C) Simulation results of concentration distribution of liquids within the micromixer (flow ratio is 1 to 1). Results show that ~ 100% mixing occurs at the channel outlet and sample becomes homogenous after 3 loops passing through the channel.
Chemical Engineering Journal 426 (2021) 130856

5

Zeng et al. [71] observed variations in the concentration profile due to rough structures in a microchannel, but for a relative roughness of 0.1 or higher. Two enzymes, carbonic anhydrase and formate dehydrogenase, were immobilised in-situ with the ZIF-8 thin film through a bio-mineralisation process as described in previous studies [28,43]. The presence of immobilised enzymes was further confirmed in fluorescence microscopy tests using fluorescence-labelled enzymes (Fig. 3). The water contact angle of the pristine micromixer surface showed that it is slightly hydrophobic (94.3° ± 4.1°) (Fig. 4A). Modification of the surface with PDA/PEI significantly improved the hydrophilicity, which reduced the water contact angle to 61.8° ± 4.2°. This is due to the abundance of amino groups present in PEI [72]. In most cases, a hydrophilic surface is favourable for the retention of enzyme activity during immobilisation as it reduces the hydrophobic interactions between enzyme and material that can cause conformational changes in enzymes [73]. The formation

Fig. 2. A, C, E) SEM images of pristine, PDA/PEI modified, and ZIF-8/CA&FDH in-situ thin film showing the growth of a highly continuous ZIF-8/CA&FDH in-situ thin film on the PDA/PEI modified micromixer surface. B, D, F) AFM images of pristine, PDA/PEI modified, and ZIF-8/CA&FDH in-situ thin film on micromixer surface showing an increase in surface roughness due to the presence of ZIF-8 microstructures.

Fig. 3. Fluorescence microscopy images in bright field and fluorescence modes of FITC-CA and atto 550-FDH immobilised in-situ in the ZIF-8 thin film.
of a ZIF-8/CA&FDH in-situ thin film on the PDA/PEI modified surface increased the water contact angle to 72.1° ± 1.5°. This is due to the increased surface roughness that can make the surface more hydrophobic, which is in agreement with the observation by Munirah et al. [43].

FTIR analysis was also conducted to examine the surface chemistry of the modified micromixer (Fig. 4B). Although there were no obvious differences between the FTIR spectra of PDA/PEI modified surface and pristine micromixer surface, the presence of PDA/PEI was previously observed from SEM and contact angle measurements. The broad absorption at 3200–3600 cm⁻¹ can be attributed to the N-H/O-H stretching vibrations of PDA and PEI [74]. The formation of ZIF-8/CA&FDH in-situ thin film on the PDA/PEI modified surface revealed a characteristic absorption peak at 421 cm⁻¹ from Zn-N stretching, indicating a reaction between Zn ions and nitrogen atoms of methylimidazole groups to form imidazolate [75]. In addition, new absorptions peaks were also observed at 660–800 cm⁻¹ which corresponds to the plane bending of the imidazole ring [76]. Furthermore, the peaks at 995 cm⁻¹ and 1146 cm⁻¹ can be attributed to the aromatic C-N stretching mode [76].

XRD analysis of the ZIF-8/CA&FDH in-situ thin film confirms that the phase structure of the composite is ZIF-8 (Fig. 4C). Distinct peaks can be observed at 20 positions of 7.4°, 10.4° and 12.7°, which are assigned to the 110, 200 and 211 planes, respectively [77]. The XRD pattern of the thin film is in good agreement with the simulated ZIF-8 pattern obtained from the database (PDF-4 database, ref ID: 00-062-1030).

3.3. Effect of liquid flow rate on biocatalytic activity

The effect of liquid flow rate on the biocatalytic activity in the micromixer was investigated. The CO₂ flow rate was fixed at 0.3 mL/min, as a relatively small adjustment was found to significantly change the two-phase flow pattern in the channel. For example, the channel was entirely filled with gas when the gas flow rate was increased to 0.5 mL/min at a liquid flow rate of 1 mL/min. Therefore, only the liquid flow rate was varied instead. The total enzyme loading in the ZIF-8/CA&FDH in-situ thin film was 0.68 mg. When the liquid flow rate was altered, the formic acid (FA) production rate after 6 h of catalysis increased from 0.37 mol/(kg enzyme h) at 0.5 mL/min to 0.97 mol/(kg enzyme h) at 1 mL/min; however, the production rate decreased to 0.70 mol/(kg enzyme h) at 2 mL/min and 0.43 mol/(kg enzyme h) at 3 mL/min (Fig. 5A). Detailed calculations of the formic acid production rate can be found in the Supporting Information (SI. Calculation of formic acid production rate). Therefore, 1 mL/min is the optimum liquid flow rate which corresponds to a Reynolds number of 6 and Dean number of 3 based on the superficial liquid velocity.

The two-phase flow pattern at the liquid flow rate of 0.5 mL/min was observed to be slug flow, which transitioned to bubbly flow at 1 mL/min (Fig. 5E). At liquid flow rates of 2 mL/min and above, the flow pattern was entirely bubbly flow. As the enzyme cascade reaction occurs in the aqueous environment [19], the lower overall biocatalytic activity observed at a flow rate of 0.5 mL/min compared to 1 mL/min could be due to the presence of long CO₂ gas slugs in the channel that hindered the reactions catalysed by CA and FDH enzymes. On the other hand, the flow rate also determines the residence time in the micromixer channel, and upon increasing the flow rate, the residence time decreases. The residence time at flow rates of 0.5 mL/min, 1 mL/min, 2 mL/min, and 3 mL/min are 3.7 min, 1.8 min, 0.9 min, and 0.6 min, respectively. While the mass transfer rate increases with increasing flow rate, this comes at the cost of a shorter residence time that can impair the conversion yield of products [78–80]. There may not be a complete, full contact between substrates and enzymes at flow rates higher than 2 mL/min, resulting in a decrease in formic acid production rate. This is similarly observed by Gong et al. [78] for the biocatalytic synthesis of isouquercitrin in a microreactor, where product yield decreased with a further increase in flow rate. In light of this, the optimum liquid flow rate of 1 mL/min was selected and used in this study.

Fig. 4. A) Water contact angle measurement of pristine, PDA/PEI modified, and ZIF-8/CA&FDH in-situ thin film showing improved surface hydrophilicity following PDA/PEI modification. B) FTIR spectra of pristine, PDA/PEI modified, and ZIF-8/CA&FDH in-situ thin film with the absorption bands corresponding to the functional groups of ZIF-8 highlighted and C) XRD pattern of pristine, PDA/PEI modified, and ZIF-8/CA&FDH in-situ thin film confirming that the composite phase structure is ZIF-8.
3.4. Evaluation of micromixer performance with benchmarks

The performance of the micromixer with threads winding around the channel was compared to a similar micromixer with smooth channel to investigate the effect of threads on the mass transfer within the channel. The micromixer with smooth channel had the same 3D helical geometry as the micromixer with threaded channel, but without the threads. The total enzyme loading in the ZIF-8/CA&FDH thin film on the micromixers with threaded and smooth channels were 0.68 mg and 0.91 mg respectively, which corresponds to immobilisation yields (enzyme loaded/enzyme added) of 16% and 22%. As shown in Fig. 5B, the formic acid production rate for the micromixer with threaded channel was significantly higher at 0.97 mol FA/(kg enzyme h) compared to the other case of 0.36 mol FA/(kg enzyme h). This can be attributed to the improved mixing of gas and liquid in the microscale due to the presence of threads, which increased the mass transfer of the CO₂ substrate and intermediate product to the enzymes. Alam and Kim [81] found that a curved microchannel with rectangular grooves at the side walls provided better mixing performance with an increase in mixing index of up to 135% compared to a curved smooth channel. Furthermore, Stroock et al. [82] also determined that grooves positioned at an angle (slanted) to the flow direction resulted in a streamline with helicoidal shape, which may further improve mixing. They demonstrated that the use of microfabrication technology can allow for precise control of the surface patterning in the microchannel, which allows the flow behaviour to be tuned to maximise mixing.

In order to elucidate the influence of ZIF-8 on the CO₂ reduction reaction, CA and FDH enzymes were immobilised on the micromixer with the threaded channel in the absence of ZIF-8. The total enzyme loading was 0.56 mg, which corresponds to immobilisation yield of 13%. The formic acid production rate after 6 h of catalysis was 0.56 mol FA/(kg enzyme h), which is lower than the case with ZIF-8/CA&FDH thin film of 0.97 mol FA/(kg enzyme h). The higher catalytic activity in the presence of ZIF-8 can be attributed to the contribution of ZIF-8 in the hydration reaction of CO₂. The imidazole group in ZIF-8 can act as a nucleophilic agent and react with CO₂ in an aqueous solution to form

Fig. 5. A) Effect of liquid flow rate on catalytic performance. 1 mL/min for the fluid has been selected as the optimum flow rate and used for further experiments. B) Comparison of formic acid yield for biocatalytic micromixer with smooth and threaded channels in the presence or absence of ZIF-8, and benchmarking of catalytic performance with a bubble column using ZIF-8/CA&FDH particles. Formic acid yield in micromixer with threaded channel is higher than the unthreaded channel, indicating the significant effects of threads on enhancement of micromixer performance. The presence of ZIF-8 also resulted in increased catalytic activity due to the synergistic effect of ZIF-8 and CA enzyme. Moreover, the proposed micromixer has three times higher product yield compared to a conventional bubble column. C) Formic acid yield of CA&FDH enzyme cascade immobilised by co-immobilisation and domino immobilisation in the micromixer with the threaded channel. D) Amount of formic acid and CaCO₃ produced using the micromixer with the threaded channel. E) Two-phase flow patterns in the micromixer at different liquid flow rates of 0.5 mL/min, 1 mL/min, 2 mL/min and 3 mL/min revealing a transition from slug flow to bubbly flow at 1 mL/min.
bicarbonate, as follows [46,83]:

\[
\text{H}_2\text{O} + \text{mLm} + \text{CO}_2 = \text{mDm}^+ + \text{HCO}_3^-(1)
\]

Therefore, there is a synergistic effect of ZIF-8 and CA enzyme to enhance the CO₂ hydration rate. The increase in CO₂ hydration rate of CA enzyme with ZIF-8 was similarly reported by Ren et al. [48] and Zhang et al. [47], where the amount of CO₂ hydrated for CA@ZIF-8 composite was up to 22 times higher compared to CA enzyme alone. Therefore, CA enzyme in the presence of ZIF-8 provides a higher concentration of soluble HCO₃⁻ substrate to the FDH enzyme, resulting in the increased formic acid yield. This is also consistent with the observation in our previous study [38].

The micromixer performance was also compared to that of a simple bubble column as benchmark (Fig. 5B), where CO₂ gas was bubbled in a solution containing ZIF-8/CA&FDH particles. The CO₂ flow rate was 0.3 mL/min, similar to that used for the micromixer. The flow pattern in the column was observed to be bubbly flow. The total enzymes loaded in the particles was 1.2 mg, with an immobilisation yield of 87%. The biocatalytic performance of the bubble column with a formic acid production rate of 0.10 mol/hr/(kgenzyme h) was significantly lower than that of the micromixers. In comparison, the use of ZIF-8/CA&FDH particles in the micromixer with threaded channel showed a production rate of 0.33 mol/hr/(kgenzyme h). The lower CO₂ conversion observed for enzymes immobilised in ZIF-8 particles compared to ZIF-8 thin film could be due to a better contact with CO₂ for enzymes localised in the film at the threaded channel area where enhanced mixing occurs. As the characteristic dimension is drastically smaller in the micromixer compared to bubble column (2.3 mm diameter micromixer channel compared to 2.5 cm diameter column), the gas-liquid interfacial area and mass transfer rates are expected to be significantly higher for the micromixer. Yue et al. [16] determined that a microchannel contactor can provide a liquid side volumetric mass transfer coefficient and gas-liquid interfacial area of at least one or two orders of magnitude higher than other gas-liquid contactors, including bubble column and packed column. This allows the cascade reaction of CO₂ to formic acid to proceed at a faster rate in the micromixer compared to the bubble column. Apart from CO₂ reduction, microchannel contactors have also been used for other gas-liquid reactions, such as halogenation and hydrogenation reactions [84–86]. The space-time yields in a gas-liquid microchannel for direct fluorination of toluene were found to be orders to magnitude higher than that of a bubble column, as miniaturisation allowed fluorination to proceed in a few seconds rather than a few hours [84].

3.5. Effect of domino immobilisation for enzymatic cascade

The effect of immobilising the CA and FDH enzymes in separate micromixers rather than co-immobilising the two enzymes in one micromixer was evaluated. As individual units of the micromixers can be connected in series easily through a tube (Fig. 1B), the CO₂ substrate can first be introduced to the CA enzyme immobilised in the first micro-mixer, and then intermediate product (bicarbonate) may continue downstream to the second micromixer with FDH enzyme for further reduction to formic acid. This enables a directional reaction sequence that follows the fluid flow. The amount of enzyme immobilised in each micromixer were 0.40 mg of CA and 0.55 mg of FDH, which corresponds to an enzyme immobilisation yield of 31.7% and 44.0% respectively. As shown in Fig. 5C, the formic acid yield observed for co-immobilisation and domino immobilisation of enzymes were quite similar. Further CO₂ hydration tests conducted on the micromixer with only the CA enzyme showed that the bicarbonate produced is two orders of magnitude higher than that of formic acid (Fig. 5D), indicating that the rate-limiting step occurs in the catalysis step of the FDH enzyme. This is supported by the observation by Zhu et al. [53] who found that the cascade bottleneck lies with the redox reaction catalysed by FDH enzyme. Oxidoreductases such as FDH function in the presence of redox cofactors like NADH as the reducing power or electron donor to the biocatalysis reaction [87,88]. The substrate and NADH bind to the active site of FDH where electron exchange takes place, reducing bicarbonate to formic acid while NADH is oxidised to NAD⁺ [19]. As the efficiency of formic acid conversion is determined by electron transfer, a possible method to improve the reduction reaction is the application of bio-electrocatalysis [89]. The cathode can supply electrons for an enhanced reduction reaction directly by enhancing the bioelectrocatalysis of FDH enzyme, or by increasing the availability of reduced NADH through regeneration [87,90,91]. Therefore, it is envisioned that the incorporation of microelectrodes in the micromixer for bioelectrocatalysis can improve the formic acid yield, which is feasible as it has been done in other similar applications such as an electrochemical microreactor for NADH regeneration [92].

Although the implementation of domino immobilisation did not yield any benefits in this case, the flexibility of the micromixers in which individual units can be easily assembled and disassembled is still of great interest because different types of enzymes can be immobilised at pre-defined locations to carry out reactions with a specific reaction order. Thus, a modular device where each module is doing its specific function while integrated can be fabricated and designed. These devices are also fabricated via 3D printing method, which allows rapid evaluation of an ideated solution. The fabrication of each device, from designing a part to performing the post-processing of the 3D printed channel, takes only less than 4 h which elucidates the huge potential of 3D printing in the fabrication of microfluidic devices. Apart from CO₂ reduction, it is envisioned that these devices can also be applied for analytical and diagnostic applications, such as quantification of lactose in milk [93].

3.6. Direct formic acid fuel cell

A long term biocatalysis experiment was conducted over 4 days in batch mode. The formic acid conversion rate started to plateau after 3 days and reached 91% after 4 days, which corresponds to a concentration of 13 mM in the Tris-HCl buffer solution (Fig. 6A). The product from the biocatalysis experiment containing formic acid and impurities (Tris-HCl, bicarbonate, NADH/NAD⁺) was then tested for electrochemical formic acid oxidation (FAO) reactions, which is a half-reaction in formic acid fuel cells. In this test, we drop-cast commercial Pt/C ink with a catalyst loading of 0.25 mg cm⁻² on a glassy carbon electrode (GCE) and employ this as the working electrode alongside a graphite rod as the counter and saturated calomel electrode (SCE) as the reference. Our bulk electrolysis results (Fig. 6B) at 0.4 V vs SCE reveal much more enhanced steady-state current density (∼0.02 mA cm⁻²) in electrolyte solution containing 0.1 M HClO₄ and products from biocatalysis experiments. Note that we apply electrolysis at this potential as the potential is higher than what is reported for Pt oxidation [59]. We conclude that this current density is arising from FAO and not water oxidation, as the background 0.1 M HClO₄ is displaying negligible current density at the same applied potential. There is a decline in formic acid oxidation activity over time as indicated by the slight decrease in current density in Fig. 6B. This may arise from the decreasing formic acid concentration as reactants are being consumed over the course of the reaction, as well as possible poisoning of the catalyst arising from CO generation and impurities present in the electrolyte solution [57,94]. While the concentration of formic acid in the biocatalysis product is limited (and hence the low current density attained), the collective results show the prospect of integrating both the systems to develop a close loop CO₂ to the energy generation system. The addition of a second metal or modifier such as Bi, Pb, Pd, or Au will likely enhance the activity of Pt catalyst towards formic acid oxidation [59,95–97]. This is believed to be due to the breach of platinum ensembles (continuous neighbouring atomic sites) that inhibits the CO poisoning effect, which can be further explored in future studies.
Fig. 6. A) Formic acid concentration in the Tris-HCl buffer solution during long term biocatalysis experiment over 4 days. B) Chronoamperometric i-t curve at 0.4 V vs SCE showing the formic acid oxidation using Pt/C catalyst.

4. Conclusion

A novel 3D micromixer with a helical shaped, threaded channel can improve the mixing and hence mass transfer of substrates to two enzymes, carbonic anhydrase (CA) and formate dehydrogenase (FDH), which are immobilised in-situ in ZIF-8 thin film on the channel. CO$_2$ and a buffer solution containing NADH were introduced into the micromixer via separate inlets, where CO$_2$ was initially hydrated to bicarbonate by CA enzyme, followed by reduction to formic acid by FDH enzyme in a cascade reaction. The liquid flow rate (1 mL/min; $R_l$ = 8; $D_l$ = 3) at which the two-phase flow pattern transitioned from slug flow to bubbly flow was found to yield the highest product conversion rate. The benefit of threads in the micromixer channel was clear, as the formic acid production rate was found to increase to 0.97 mol$_{FA}$/kg$_{enzyme}$h from 0.36 mol$_{FA}$/kg$_{enzyme}$h for a smooth channel. In addition, benchmarking with a conventional bubble column also showed a higher formic acid yield for the micromixer with threaded channel by three folds. As individual enzymes can be immobilised in separate micromixer units and assembled in series, this also enabled reactions with a specific reaction order to take place. We also present an initial proof of concept for the development of a closed-loop CO$_2$ action order to take place. We also present an initial proof of concept for the development of a closed-loop CO$_2$ to energy generation system, by grading these two systems for green energy generation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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