Lag Time Spectrophotometric Assay for Studying Transport Limitation in Immobilized Enzymes

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ABSTRACT: Enzymes are promising catalysts for bioprocessing. For instance, the enzymatic capture of CO2 using carbonic anhydrase (CA) is a carbon capture approach that allows obtaining bicarbonate (HCO3−) with no high-energy input required. However, application in a commercially viable biotechnology requires sufficient enzymatic lifetime. Although enzyme stabilization can be achieved by different immobilization techniques, most of them are not commercially viable because of transport limitations induced by the immobilization method. Therefore, it is necessary to develop assays for evaluating the role of immobilization on transport limitations. Herein, we describe the development of a fast and reproducible assay for screening immobilized CA by means of absorbance measurement using a computer-controlled microplate reader in stop–flow format. The automated assay allowed minimizing the required volume for analysis to 120 μL. We validated the assay by determining lag times and activities for three immobilization techniques (modified Nafton, hydrogels, and enzyme precipitates), of which linear polyethyleneimine hydrogel showed outstanding performance for CA immobilization.

INTRODUCTION

Bioprocessing has been an expanding field over the last couple of decades with increasing industrial use, including in the pharmaceutical industry, biofuels industry, and bioremediation. Most bioprocessing is subdivided between cell-based and cell-free bioprocessing. Cell-free bioprocessing has lagged behind cell-based bioprocessing because of stability issues of the enzymes utilized, and the need for immobilizing them on a support for flow-through reactors. Furthermore, besides the bioprocessing field, enzymes have gained critical importance for different clinical and biosensing applications. A wealth of new techniques for immobilizing and stabilizing enzymes have been demonstrated, which are typically evaluated with specific activity assays, protein leaching assays for evaluating immobilization, and long-term activity assays for evaluating stability. These assays provide proof-of-concept knowledge about the effectiveness of the immobilization techniques. However, no information about whether the technique can be used in an industrial reactor is obtained, because they do not address transport limitations that are frequently introduced by the immobilization technique. Additionally, the majority of these assays are not automated, or introduce variables, such as the response time of a pH electrode or the need of temperature control to slow the reaction rate. Therefore, in this paper, we detail an automated stop–flow spectrophotometric assay that can be performed at room temperature for evaluating transport limitation in enzyme immobilization systems.

Carbonic anhydrase (CA) was utilized as our model enzyme. CA (EC 4.2.1.1) is a zinc-containing metalloenzyme, which catalyzes the reversible hydration of CO2 with uncommonly high turnover rate, up to 106 per second, according to the following reaction:

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+ \tag{1}
\]

CAs have an apparent molecular weight of 29–55 kDa. Naturally, CAs contribute to several biological processes such as acid–base balance, carbon dioxide transport, ureagenesis, photosynthesis, and they are produced by different tissues. The use of CA as a biological catalyst for capturing CO2 is an extremely interesting approach, which would minimize excessive energy requirements. Furthermore, the development of a biotechnology based on an enzymatic catalyst to convert CO2 directly to bicarbonate (HCO3−) does not require high-energy input at all. Immobilization techniques have been shown to greatly increase the stability and lengthen the half-life of different enzymes, including CA. However, performance can be strongly affected by transport limitations because of the
very high turnover rate of CA. It is easy to be limited by transport of reactant and/or product within the immobilization matrix, if the immobilization technique retards transport. Thus, CA is a promising candidate to be used as a model enzyme in this study.

The catalytic activities of CAs are usually determined using a pH assay, known as the Wilbur−Anderson (W−A) assay, which relies on the change in pH obtained because of an increase in H⁺ concentration as indicated in eq 1, when the reaction proceeds to equilibrium.7 However, the W−A assay requires temperature control to slow the reaction rate and allow for pH monitoring. Accordingly, the assay is commonly performed maintaining the temperature between 0 and 4 °C. Moreover, as a fast response is needed, the employed pH electrode must have a short response time and small dimensions to contain the volume for the assay at a reasonable amount. CA activity can also be measured by spectrophotometric analysis, following the increase of absorbance at 348 nm using p-nitrophenyl acetate as an alternative substrate for the enzyme, with no temperature control required.8 Because of the uncommonly high turnover of CA, however, this analysis does not allow us to differentiate between activity losses because of denaturation or limiting the degrees of freedom of the enzyme and apparent activity losses because of slow transport of reactant and/or product over long length scales in the immobilization matrix.

Inspired by the possibility to measure CA activity by spectrophotometric analysis, herein, a spectrophotometric assay was developed, which determined the catalytic activity by remote control, minimizing the reaction volume to 120 μL at ambient temperature and ensuring a controlled time between substrate injection and absorbance measurement for lag-time-based transport-limitation studies. Furthermore, the Synergy HTX Multi-Mode Reader utilized for the assay allows temperature control up to 50 °C, thus being applicable also for catalytic activity studies of thermophilic enzymes. The influences on activity and lag-time performances for three immobilization techniques were determined, specifically magnetically separable enzyme precipitate coating (Mag-EPC),9 linear polyethyleneimine (LPEI) hydrogels,10 and tetrabutylammonium bromide (TBAB)-modified Nafion.11

The lag time assay allows for the determination of the time it takes substrate to get to the immobilized enzyme, the substrate to react, and the product to diffuse away. Therefore, if the immobilization technique retards transport or requires long transport distances to the enzyme, the lag time will be significantly larger than if there is facile diffusion and a short diffusion length to the enzyme. It has to be noted that the immobilization strategies were specifically chosen as model immobilization systems. We expect TBAB-Nafion immobilization strategies to decrease activity because of the polymer being dense and retarding transport. In the case of the Mag-EPC immobilization system, the use of the cross-linker will likely decrease the activity of the CA. Immobilization with LPEI hydrogel was selected because of the polymer’s highly cationic polyamine backbone, being largely protonated under neutral conditions,12 which might establish a favorable interaction with the anionic surface of the immobilized enzyme.

**RESULTS AND DISCUSSION**

Representative absorbance increases investigated using the developed time-dependent spectrophotometric assay for free CA in solution, CA immobilized in LPEI hydrogels, CA immobilized via Mag-EPC, and in TBAB-modified Nafion are shown in Figure 1 (see the Methods section for spectrophotometric assay and preparation details). The evolution over time...
of the absorbance was determined versus the results obtained in the control experiment, where no enzyme was present (Figure 1, black dots). It has to be noted that the enzyme dynamics might be influenced differently in every replicate because of the immobilization step, making it of critical importance to perform several independent replicate experiments. Accordingly, four independent replicate experiments were performed for all the cases under investigation, allowing for the determination of activity and lag times (Figures 2 and 3, respectively).

![Figure 2](image2.png)
**Figure 2.** Activity measurements for the three immobilization techniques (LPEI, TBAB-Nafion, and Mag-EPC) and for the enzyme in solution.

![Figure 3](image3.png)
**Figure 3.** Lag-time results for the three immobilization techniques (LPEI, TBAB-Nafion, and Mag-EPC) and for the enzyme in solution.

As expected, the free enzyme in solution retained the highest activity (Figure 2), with $16.9 \pm 0.4$ units $\times 10$ mg enzyme$^{-1}$. Immobilization using LPEI resulted in the smallest loss of enzyme activity, obtaining a value of $11.7 \pm 0.2$ units $\times 10$ mg enzyme$^{-1}$. LPEI is a biocompatible hydrogel, so it is expected that it would not confine the enzyme or retard transport. On the contrary, immobilization using TBAB-Nafion and Mag-EPC caused a significant loss in activity, with values of $6.1 \pm 0.7$ and $6.4 \pm 0.9$ units $\times 10$ mg enzyme$^{-1}$, respectively. However, the standard activity assay does not differentiate whether the loss in activity is due to retarded transport through a dense matrix or a decrease in activity because of denaturation or decreased degrees of freedom during cross-linking or encapsulation.

Utilizing an automated addition of substrate with an absorbance reading every 0.09 s, it was possible to determine the lag time for the conversion of the substrate by CA immobilized with the three different techniques and the free enzyme in solution. CA immobilized using LPEI allowed achieving the lowest lag time as shown in Figure 3, requiring only $4.4 \pm 0.4$ s to initiate the conversion of the substrate. Free CA in solution and Mag-EPC showed similar performances with lag times of $7.3 \pm 0.7$ and $7.6 \pm 0.9$ s, respectively. Immobilization with TBAB-modified Nafion produced the lowest performances, with a lag time of $10.8 \pm 0.3$ s. The outstanding performances of LPEI-immobilized CA might be related to the electrostatic interaction between the anionic surface of the immobilized enzyme and the cationic polyamine backbone, which allowed for maintaining the enzyme at the interface with the solution, minimizing diffusion effects and possibly facilitating the diffusion of the substrate to the active site of the enzyme. Further studies will be required to specifically determine the origin of the decreased lag time for CA immobilization in the LPEI polymeric layer. On the contrary, when TBAB-modified Nafion was used for the immobilization, the lowest activity was obtained together with the highest lag time. A limited hydration of the dense Nafion polymeric layer might be responsible for this performance, together with a local decrease of pH because of protons' retention inside the polymeric layer. In fact, LPEI-based polymers have been reported to present a “proton pump effect” because of the ability of corresponding cross-linked films to exchange protons with the bulk solution. This effect might facilitate the fixation of carbon dioxide inside the hydrogel, by removing the obtained protons and maintaining the optimal environment for CA, which has been reported as slightly alkaline for immobilized CA. On the contrary, TBAB-Nafion does not present the “proton pump effect”; thus, an increased concentration of protons inside the layer might decrease the pH locally and inhibit the activity of CA. Mag-EPC-immobilized CA showed a considerable loss of activity; however, a lag time similar to the free enzyme in solution was obtained. This result can be explained considering that, conversely from LPEI and TBAB-Nafion immobilization, the Mag-EPC immobilization system does not introduce a polymeric layer that can positively (or negatively) influence the transport of chemical species, resulting in a lag time similar to the free enzyme. Taking into consideration the extended lifetime reported for CA immobilized in Mag-EPC with the half-life of 236 days, this immobilization would be of extreme interest for application where recovery of the enzyme is necessary, as the dispersed particles can be magnetically captured, simplifying the recovery process.

The developed spectrophotometric assay allowed an easy determination of lag times and enzymatic activities at room temperature for the three immobilization techniques. The two parameters together allowed for a comprehensive evaluation of the influence of the different immobilizations on the catalytic performances of CA, remarking on the role of the hydrophilic properties and “proton pump” effect of LPEI hydrogels for outstanding performance. Accordingly, the developed spectrophotometric assay allowed a deeper insight of the catalytic performances of CA in the three immobilization techniques than the standard W–A assay. Moreover, the operational time required for the W–A assay is much higher, making this assay not suitable for an elevated number of samples. It has to be noted that a better understanding of catalytic performance for immobilized enzymes is critical both from a scientific point of view, in order to clarify the specific influence of immobilization techniques on enzymatic response, as well as for clinical, pharmaceutical, and other industries involved in bioprocessing. Different immobilization techniques might be designed for a better control of enzymatic cascade reactions depending on the
lag times for specific immobilized enzymes. Additionally, the developed assay would benefit the field of biosensors, where lag times might influence response time and lead to erroneous measurements.

**CONCLUSIONS**

The stop–flow spectrophotometric assay allowed for the easy, fast, and reproducible determination of lag-time and catalytic activity parameters of CA immobilized with different techniques. The assay was performed at room temperature (20 ± 2 °C), with a reaction volume of only 120 μL, which allowed for minimizing the amount of immobilized enzyme and a consequent decrease of the assay cost. Depending on the desired operational conditions, the three immobilization techniques showed interesting features. LPEI hydrogels could be successfully utilized to immobilize CA when fast CO₂ fixation is required. On the contrary, Mag-EPC can be successfully applied in long-term experiments because of the remarkable half-life reported and comparable lag times to free enzyme catalytic activity. The developed assay will be beneficial for the continuous development of enzymatic systems such as the one presented here for CO₂ fixation, as well as for fundamental studies, which are critical for the successful development of enzymatic biotechnology and their implementation in industrial systems.

**METHODS**

**Chemicals.** CA from bovine erythrocytes, TBAB, p-nitrophenyl acetate, Nafion, acetonitrile, poly(ethylene glycol) diglycidyl ether, aniline, 3-aminobenzoic acid, glutaraldehyde, and ammonium sulfate were purchased from Sigma (St Louis, MO, USA). Sodium phosphate monobasic was purchased from Fisher Chemicals.

**CA Immobilization Techniques. Immobilization by Mag-EPC.** CA was immobilized on carboxylated polyaniline nanofibers (cPANFs) via the approach of Mag-EPC, as described elsewhere. Briefly, the carboxyl groups of cPANFs were modified by 1-ethyl-3-(3-(dimethlamino)propyl) carbodiimide hydrochloride (10 mg mL⁻¹) and N-hydroxysuccinimide (50 mg mL⁻¹) in 100 mM 2-(N-morpholino)ethanesulfonic acid buffer (pH 6.0). A solution of CA (10 mg mL⁻¹) in 100 mM sodium phosphate buffer (PB, pH 7.6) was mixed with the modified cPANF and incubated at room temperature for 2 h. Subsequently, amine-functionalized magnetic nanoparticles and ammonium sulfate were added to the mixture. After 30 min of incubation, glutaraldehyde solution was added at a final concentration of 0.5% (w/v), followed by the incubation at 4 °C for 17 h. Unreacted glutaraldehyde was quenched by 100 mM Tris-HCl buffer (pH 7.6). After excessive washing, the Mag-EPC was stored in 100 mM PB (pH 7.6) at 4 °C. The sample of Mag-EPC-immobilized CA was diluted 33 times and 10 μL of the dispersion was added to the well, for a final content of immobilized CA of 3 × 10⁻³ mg.

**Immobilization on TBAB-Modified Nafion.** TBAB-modified Nafion was prepared by adding the TBAB to a 5% by weight Nafion suspension as previously reported, and salt-extracting the excess TBAB and HBr. In a 500 μL tube, 1.5 mg of CA was added to 75 μL of 100 mM PB (pH 7.6). Twenty-five microliters of the TBAB-modified Nafion were added to the tube and mixed by vortex for 1 min. Subsequently, the suspension was sonicated for 15 min and diluted 50 times using 100 mM PB (pH 7.6). Ten microliters of the suspension were added to the well and let to dry for 1 h to ensure evaporation of the solvent. The final content of immobilized CA was 3 × 10⁻³ mg.

**Immobilization of LPEI Hydrogel.** For the LPEI immobilization, in a 500 μL tube, 14 μL of a 1% by weight LPEI suspension (prepared as discussed in ref 12b) was added to 6 μL of a 10 mg mL⁻¹ solution of CA in 100 mM PB (pH 7.6). Subsequently, 0.75 μL of a 10% by volume solution of poly(ethylene glycol) diglycidyl ether was added to the tube and diluted 10 times using 100 mM PB (pH 7.6). Ten microliters of the suspension were added to the well and let to dry for 1 h to ensure evaporation of the solvent. The final content of immobilized CA was 3 × 10⁻³ mg.

**Activity and Lag-Time Spectrophotometric Assay.** The automated stop–flow spectrophotometric assay for enzyme catalytic activity was developed using a microplate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc.). A 96-well plate is utilized for enzyme immobilization during the assay, which allows for performing independent replicate experiments simultaneously. Utilizing the BioTek Gen5 2.07 data analysis software, the assay is designed to perform substrate addition by remote control, measuring the increase in absorbance every 0.09 s immediately after the additions. The assay does not require maintaining the temperature at 0–4 °C to slow the reaction rate, and can be executed directly at room temperature. The absorbance can be measured for the desired reaction time, depending on the enzyme catalytic activity to be determined. Lag times are determined as the time required before substrate conversion started (i.e., slope started increasing, as shown in Figure 4), indicated by an increase of absorbance compared to the control experiment performed in the absence of enzyme. Enzymatic activities were calculated from the increase of absorbance per second as described in the calculation section. A schematic for the assay procedure is shown in Figure 4.
Specifically, in the case of CA catalytic activity study, the absorbance was monitored at 348 nm over 45 s of reaction time at room temperature (20 ± 2 °C). The substrate utilized for the analysis was 60 mM p-nitrophenyl acetate (10.9 mg mL⁻¹) in acetonitrile as solvent. Ten microliters of immobilized enzyme, prepared as previously described, were placed for each well of the 96-well plate. All the samples were diluted to 100 μL using PB (pH 7.6). Subsequently, 20 μL of substrate was added to the well by means of a remote-controlled pump. After addition, the well was immediately shaken for 2 s at 731 cycles-per-minute. Following the shaking step, the absorbance at 348 nm was measured for 45 s. All the analyses were performed with four replicates. The obtained values of activity and lag time were compared to the results obtained following the same procedure but using a solution of CA in 100 mM PB (pH 7.6) (enzyme not immobilized).

**Calculations.** The concentration of p-nitrophenyl acetate converted was calculated using the Lambert–Beer law:

\[
A_{\text{min}} = \varepsilon \cdot c \cdot l \cdot \frac{m}{e}
\]

where \( \varepsilon \) is the molar absorptivity constant for p-nitrophenyl acetate at 348 nm (400 M⁻¹ cm⁻¹), \( l \) is the length of the cuvette (1 cm), \( A_{\text{min}} \) is the slope of the absorbance per minute as obtained during the measurement, and \( c \) is the concentration of the product. The activity of the enzyme is indicated as units \( \times 10 \text{ mg enzyme}^{-1} \), thus representing the amount of substrate in μmol, converted by 10 mg of enzyme (immobilized or in solution).

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**Notes**

The authors declare no competing financial interest.

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