Immobilization of carbonic anhydrase in a hydrophobic poly(ionic liquid): A new functional solid for CO_2 capture

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ABSTRACT:

Poly(ionic liquid)s are interesting materials for CO_2 separation due to their affinity towards CO_2. Carbonic anhydrase, as an enzyme that catalyzes the hydration reaction of CO_2, represents tremendous opportunities for enhanced CO_2 capture in aqueous solutions. Yet, when used as a free enzyme solubilized in the liquid medium, it has limited practicality. Here, we report the study on carbonic anhydrase immobilization in poly(ionic liquid)s by entrapment. Due to limited enzyme solubility in the hydrophobic ionic liquid monomer solution, carbonic anhydrase was suspended instead of solubilized prior to bulk polymerization. The immobilization protocol was optimized to maximize the catalytic efficiency of the resulting solid by evaluating the effect of lyophilized suspension hydration and particle size. Immobilized carbonic anhydrase properties were investigated by measurement of kinetic parameters, reusability, thermal and storage stability. Using p-NPA hydrolysis as a model reaction, kinetic parameters were 0.22 s^{-1} mM^{-1} (kcat/Km) for free enzyme and 0.06 s^{-1} mM^{-1} for the immobilized biocatalyst, indicating a decrease of activity upon

immobilization. Immobilized enzyme, however, presented better storage stability than free enzyme as after one month the activity did not change. In the case of dissolved carbonic anhydrase, it decreased by about 30%. The entrapped enzyme could be utilized in 5 consecutive cycles of CO_2 hydration while keeping a relative activity superior to 60%.

INTRODUCTION

Carbonic anhydrase (CA) is a metalloenzyme that uses CO_2 as substrate[1], see the reaction below. The urgent need for solutions to reduce net CO_2 emissions and utilization of CO_2 as a resource[2,3] has encouraged the study of CA in post-combustion CO_2 capture processes[4–6]. Most works focused on CA free in solution, i.e., dissolved[7–13]. However, considering the harsh conditions to which the enzyme would be exposed in post-combustion CO_2 capture, the utilization of free enzymes is generally less convenient[5]. Consequently, current research is addressing CA immobilization in/on different supports and following different immobilization routes as it is a common approach to increase enzyme stability and allow enzyme reutilization[5,14–18].

$$CO_2 + H_2O \overset{CA}{\leftrightarrow} HCO_3^- + H^+$$

Ionic Liquids (ILs) are a group of low melting point (<100°C) organic-inorganic salts with a wide range of applications including biotechnology and CO_2 separation. ILs have been extensively studied as media for enzymatic reactions[19–23]. The primary interest is to replace organic solvents. These have been used in biocatalyzed reactions whose equilibrium conversion is compromised by water presence or whose reactants have limited water solubility[19,24]. When compared to biocatalysis in organic media, IL based biocatalysis has provided improvements in activity, stability and selectivity[19,21,24]. It is difficult to draw general conclusions regarding the compatibility of ILs and enzymes as it depends on every pair, IL concentration and co-solvent presence. For example, lipases have displayed better activities in pure imidazolium based ILs with hydrophobic anions such as [BF4] and [PF6] than those having hydrophilic anions, $[CH_3CO_2]$, $[NO_3]$,$[CF_3CO_2]$, $[CH_3SO_3]$ or [Cl][25–27]. In aqueous solutions, imidazolium cations are more prompt to lipase activity reduction and destabilization than ammonium and cholinium based ILs, which in some cases lead to enzyme activation[28,29]. However, this trend is not consistent for tyrosinase enzyme[28]. The enzyme stem bromelain retains good activity in 0.1 M solution of hydrophilic ILs such as [BMIm][Br], [BMIm][Gly], [Ch][Br] and [Ch][Gly]. Interestingly, it presents better activity and stability in imidazolium and bromide based ILs than in those identified as biocompatible[30,31]. Cellulases were investigated in a series of hydrophilic and hydrophobic ILs displaying activity only in [MIm][Cl] and $[HEMA][CH_3OSO_3]$ [32]. The enzyme presented activity and good stability in the later despite of being

hydrophilic. However, other authors showed that imidazolium based ILs with anions [DMP], [Cl] and [CH_3CO_2] resulted in an activity reduction of cellulase up to 85%[33]. CA incorporation into ILs has received little attention. So far, it was applied in conjunction with ILs in the preparation of supported IL membranes for CO_2/N_2 separation. CA was first investigated into the hydrophobic IL [BMIm][NTf2][34–36]. A minimum water activity in the IL was requited in order to display enhancements in CO_2 permeability (~20-100%). In a more recent study, CA was dissolved in an aqueous solution of [Ch][OH][37]. CA produced minor improvements in CO_2 permeability. CO_2 absorption experiments showed that the absorption rate is slightly improved by CA. Better results were obtained when glycerol was added in large quantities (75% $_{w/w}$) to stabilize the enzyme.

Furthermore, several works have explored the immobilization of enzymes in ILs derived materials[38,39]. Enzyme immobilization in ILs can be accomplished by incorporating the enzyme to an IL monomer (IL with polymerizable groups) solution which is polymerized to form a poly(ionic liquid) (PIL)[40–46]. Another route is to immobilize the enzyme in an IL that is solid at room temperature, forming the so-called IL-coated enzymes. In this case, it is required to melt the IL before enzyme addition and to solidify it afterwards by cooling[47–49]. Too thick solid IL coatings were associated to diffusional mass transfer limitations. More complex procedures were developed later to prepare IL-coated enzymes by co-lyophilization or co-precipitation of enzyme-IL aqueous solutions[49,50]. Other methods than entrapment have also been employed such as enzyme adsorption on IL modified mesoporous silica SBA-15[51–53], PIL-graphene composites[54], IL modified magnetic nanoparticles[55,56], IL modified chitosan-coated[57–59], cellulose-coated[60], poly(dopamine)-coated[61] and silica-coated magnetic nanoparticles[62] and IL functionalized gold nanoparticles[63]. Strong enzyme-support attachment can be achieved by means of covalent bonding[5]. Examples include amino-functionalized ILs which were grafted on magnetic nanoparticles[56] or deposited on an electrode for the development of biosensors[64], IL-cellulose films[65], IL-graphene composites[66], epoxy functionalized PILs-coated magnetic nanoparticles[67], porous silica materials bearing ILs with a carboxyl group[68–70], carbographene oxide biosensors modified with aldehyde functionalized ILs[71] and chitosan-IL-$CuPc^S$ biosensors prepared by LbL assembly[72].

ILs and PILs have also been thoroughly studied for gas separation using selective membranes and physical/chemical absorption due to their preferential interaction with CO_2[73–75]. A limited number of works focused on promoting ILs with CA[34–37,76]. Most of them investigated hydrophobic ILs. However, PILs can absorb larger quantities of CO_2 than non-polymerized ILs and with faster absorption kinetics[77].

Considering the good interaction between PILs and CO_2 and that CO_2 is the substrate of CA, it might be interesting to immobilize CA in these materials. This article also proposes an innovative way of enzyme immobilization. In this work, bovine origin CA was suspended in a hydrophobic IL monomer mixture and photopolymerized to prepare CA entrapped PILs (CA-PILs). This methodology allows increasing the enzyme loading much more than could be possible by dissolving the enzyme. Despite of producing a less homogenous catalyst (poorer enzyme dispersion), the solid material was expected to display higher activity per g. This is important if it is considered that the final price of an immobilized enzyme is heavily affected by the price of the support (especially for cheap enzymes)[78]. Based on the general trends of enzyme activity/stability in ILs[79] and PIL CO_2 permeability[80,81], the hydrophobic IL 1-vinyl-3-hexylimidazolium bis(trifluoromethylsulfonyl)imide ([VHIm][NTf_2]) was selected as monomer. ILs are generally hygroscopic, even hydrophobic ILs absorb water[82,83]. Therefore, it was expected to provide to the enzyme the required amount of water. The hydrophilic IL monomers, 1-vinyl-3-hexylimidazolium bromide ([VHIm][Br]) and 1-vinyl-3-ethylimidazolium bromide ([VEIm][Br]) were evaluated as comparison. In addition, the polymer was cross-linked with an ethylene glycol derivative. Ethylene glycol shows water affinity, it is readily soluble in ILs and it is enzyme compatible[84,85]. In this work, CA-PILs reusability, thermal and storage stability, and CO_2 hydration and p-NPA hydrolysis activities were measured and compared to the free CA in solution.

MATERIALS AND METHODS

CHEMICALS

Lyophilized carbonic anhydrase from bovine erythrocytes, 1-vinylimidazole (≥99%), 1-bromohexane (98%), bis(trifluoromethylsulfonyl)imide lithium salt (>99.0%), anhydrous acetonitrile (99.8%), diethyl ether (99.7%), 2-hydroxy-2-methylpropiophenone (97%), tri(ethylene glycol) divinyl ether (98%), 4-nitrophenol (≥99%), 4-nitrophenyl acetate (p-NPA), tris(hydroxymethyl)-aminomethane powder (≥99.9%) and Bradford reagent were all obtained from Sigma-Aldrich. Ethyl Acetate (99.8%) was purchased for VWR. Rain-X, was acquired from Wynn's. All of them were directly used without any further purification.

IL monomer production and characterization

[VEIm][Br], [VHIm][Br] and [VHIm][NTf_2] were produced as reported elsewhere[81,86]. Their chemical structure and their purity were characterized using ^{1}HNMR and ^{13}CNMR in $CDCl_3$ ([VHIm][NTF2]) or DMSO-d_6 ([VEIm][Br]/[VHIm][Br]) (Bruker Avance 500 spectrometer, Bruker) and FT-IR spectroscopy (Thermo Nicolet Nexus

870 FTIR coupled with a Continuum microscope, Nicolet). Characterization results are included as Supporting information (Figures S1-S6).

ENZYME IMMOBILIZATION

In order to prepare CA-PILs, first a solution was prepared containing all chemicals required for the polymerization. Typically, 1 g of [VHIm][NTf_2] was placed in a 10 ml vial with a magnetic stirrer. Then, tri(ethylene glycol) divinyl ether (cross-linker) was added to have 10% $_{mol/mol}$ with respect to monomer. Finally, 2-Hydroxy-2-methylpropiophenone (free radical initiator) was added to have 0.5% $_{w/w}$ with respect to the total. The solution was stirred until it was homogeneous. In a separated 10 ml vial, between 2 and 5 mg of lyophilized CA were carefully placed. Next, the polymerization solution was added to have 20 mg CA/g of solution. While being agitated gently, the CA suspension was humidified by introducing a tube in the vial through which humidified nitrogen flowed (250 ml N/min, bubbled in UP water at room temperature) during 1 h. Some samples were not humidified to study the effect of humidification. Then, the humidified suspension was sonicated during a given time period (0-75 min) in a cold-water bath (the temperature was kept below 20 °C). 10-20 µl of the sonicated suspension were transferred to a hydrophobically treated glass plate and sandwiched with a smaller microscope glass slide also hydrophobically treated. A constant weight was placed during a few seconds on top of the glass slide to ensure a better distribution of the suspension and a homogenous thickness. Subsequently, polymerization was carried out using a 368 nm UV lamp (MINILYNX 20W E27 BL368, Sylvania) during at least 30 min at room temperature. Afterwards, the glass slides were detached and polymeric films recoded using a razor blade. The polymer films were easily peeled off from the glass slides thanks to the hydrophobic treatment. The treatment was performed as follows: to deposit a thin layer of hydrophobic coating in both glass surfaces, they were sprayed with Rain-X. The product was homogenously distributed on the glass surface using a lint free paper and it was subsequently dry[87]. Finally, the recovered PIL films with immobilized CA were grinded manually with a mortar and stored in the fridge until use. The whole process is schematized in Figure 1.

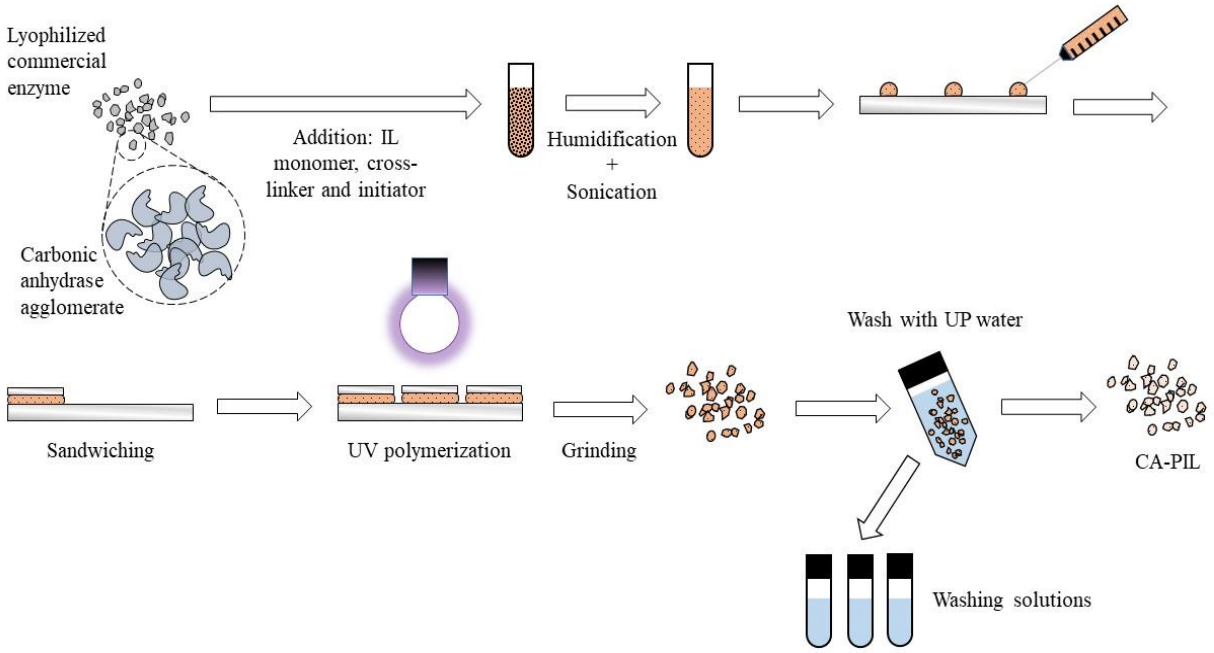

Figure 1. Graphic summary of CA-PIL preparation.

CA-PILS CHARACTERIZATION

CA-PILs were characterized by SEM (Ultra 55 Feg Sem, Zeiss), EDX (Quantax, Bruker), ATR-FTIR spectroscopy (Equinox 55, Bruker) and optical microscopy (Olympus AX70 microscope equipped with a digital camera, CMEX 5). SEM samples were studied using an acceleration voltage of 5 kV after been gold sputtered (Quorum Q150 RS) while those for EDX were carbon coated. Raw IR spectra were treated with the ATR correction. EDX results are included as Supporting Information (Figure S7). TGA analysis (TGA/SDTA 851e, Mettler Toledo) were conducted from 30 to 900°C at a heating rate of 10K/min under N_2 atmosphere (50 ml/min).

ENZYME ACTIVITY

Enzyme activity was measured using the p-NPA hydrolysis assay[88,89]. First, a known amount of enzyme (free or immobilized) was added to 2.7 ml of aqueous Tris-HCl buffer (7.5 pH, 50 mM). The enzyme was incubated during 5 min at 20-21 °C before adding 0.3 ml of a 10-50 mM p-NPA solution in acetonitrile. Similarly to other authors[15,18,90–93], after a given amount of time, a sample was withdrawn from the reaction solution and its absorbance was measured at 400 nm (absorbance maximum) using a UV-VIS spectrophotometer (DR/4000U, Hach). The transfer of the sample to the spectrophotometer was done rapidly (less than 10s) to ensure minimal increase in the absorbance. The activity was determined from the absorbance increase rate expressed as arbitrary absorbance units per minute. Immobilized

CA activity is compared to free CA in solution using the efficiency[94], defined as the ratio of immobilized enzyme specific activity over the free enzyme specific activity:

$$Efficiency = \frac{U_{immobilized}}{U_{free}} \quad (1)$$

$$U = \frac{r - r_{blank}}{C_{CA}} \quad (2)$$

Where U is the specific activity in arbitrary absorbance units/min mg CA mL^{-1}, r is the observed initial rate of reaction (biocatalyzed + non-catalyzed reaction), r_{blank} is the blank rate (both in arbitrary units/min) and C_{CA} (mg CA/mL) is the actual amount of enzyme in the reaction medium (free or in the form of CA-PIL) divided by the reaction volume. To eliminate the effect of improperly entrapped CA, before every activity test the required amount of CA-PIL was washed three times with 3 ml of ultrapure water. Washing water was carefully stored to be analyzed afterwards by Bradford assay[95]. Due to the hydrophobic nature of the IL, unreacted monomer is unluckily removed by water. Then, the actual enzyme loading (mg enzyme/g CA-PIL) was determined using the following expression:

$$Enzyme\ loading = \frac{m_{CA0} - \sum VC}{m_{CA-PIL}} \quad (3)$$

Yield and activity recovery, as defined by[94], were estimated with the equations below.

$$Yield = \frac{m_{CA0} - \sum VC}{m_{CA0}} \quad (4)$$

$$Activity\ recovery = Efficiency * Yield \quad (5)$$

In both expressions m_{CA0} is the initial amount of enzyme included in the CA-PIL formulation in mg, V is the volume of washing solution in ml, C is the CA concentration in a given washing solution determined by Bradford assay (mg CA/ml) and m_{CA-PIL} is the CA-PIL mass in g. C_{CA} in equation 2 was computed using enzyme loading.

CO_2 HYDRATION ACTIVITY

CO_2 hydration catalysis was studied by a modified delta pH assay[96], originally developed by Wilbur and Anderson[97], at 4°C. A known amount of CA-PIL was placed in a stirred beaker with 5 ml of Tris-HCl buffer (pH 8.3 at 25 °C and 20 mM). Next, 4 ml of CO_2 saturated solution were added. The latter solution was prepared by bubbling CO_2 during

at least 1 h and 30 min in 250 ml of UP water. During absorption, the solution was placed in a cold-water bath (2-4°C). After absorption, it was let to rest during at least 30 min properly closed in the same bath. Once the CO_2 saturated solution was added to the buffer, the subsequent pH drop was measured and recorded (914 pH/Conductometer, Metrohm). Blank experiments of delta pH assay allowed to subtract the non-catalyzed hydration reaction. Free enzyme hydration tests were also performed as comparison. CO_2 hydration activity, U_{CO2}, is expressed in Wilbur-Anderson units per mg of CA, WAU (see Eq. 6). To prevent misleading results because of leaching, CA-PILs were washed 3 times with 3 ml of buffer before the assay. Washing water and the reaction medium after the test has finished were stored and analyzed by the Bradford assay.

$$U_{CO2} = \frac{\frac{T_b - T_{CA}}{T_{CA}}}{m_{CA}} \qquad (6)$$

Where T_b and T_{CA} are the average times for blank and CA catalyzed assay, respectively, required to decrease the pH from 8.3 to 6.3 in s.

REUSABILITY

CA-PILs reusability was studied via p-NPA hydrolysis and CO_2 hydration. Once a given amount of PIL-CA was tested, the immobilized enzyme was separated by centrifugation and washed three times with buffer before doing another test[98]. The change in enzyme activity throughout the cycles was expressed in the form of retained activity, R. The retained activity is defined as the ratio of observed activity at n^{th} cycle (U_n) and initial activity (U_o):

$$R = \frac{U_n}{U_o} \qquad (7)$$

THERMAL STABILITY

The effect of immobilization in CA thermal stability was also measured by the p-NPA hydrolysis test. A certain amount of CA (free or immobilized) was placed in a stirring vessel with 3 ml of UP water and incubated at the desired temperate (20, 30, 40, 50, 60, 70 °C) during 1 h. Then, it was immediately placed in a cold bath (0-4°C) during 5 min. After at least 15 min at room temperature, the activity was measured. CA-PILs were washed twice with ultrapure water before the activity assay. Washing and incubation water were analyzed to determine enzyme leaching and enzyme loading. The activity is expressed in relative units in which the reference is the optimum temperature for free and immobilized enzyme.

STORAGE STABILITY

CA (free or immobilized) was incubated statically at 21 °C in 0.1 M Na_2CO_3 during 4 weeks. Before measuring the activity, CA-PIL was washed twice with UP water and its activity was determined using p-NPA as explained before. The incubation solution and washing water were analyzed to determine enzyme leaching and actual enzyme loading during the activity test.

RESULTS AND DISCUSSION

PRELIMINARY ACTIVITY RESULTS

In preliminary experiments, the solid biocatalyst was prepared simply by mild agitation of the CA-IL monomer suspension and subsequent photopolymerization. The efficiency was low (lower than 1%) and a loading of at least ~10 mg CA/g PIL was required to show activity (see Supporting information, Figure S8). The PIL support without enzyme did show any catalytic activity. Exposure to UV light did not show any detrimental effect in free CA activity (see Supporting information, Figure S9) therefore it was unlikely to cause any reduction in the efficiency of the immobilized CA. To improve the activity, the preparation protocol was modified.

EFFECT OF ENZYME HYDRATION

Complete hydration and dissolution of enzymes is not a requirement for displaying catalytic activity. This feature has been exploited in non-aqueous biocatalysis where enzymes are suspended instead of being dissolved[99,100]. This generally results in lower activity than aqueous solution[101,102]. Some authors highlighted that enzyme suspension hydration resulted in enzyme activity improvements[101,102]. By analogy, in this work, CA was added in the form of lyophilized powder that was dispersed in the monomer solution to form a suspension before polymerization. In order to see the effect of enzyme hydration and enzyme microenvironment humidification, CA-PILs were prepared according to the procedure already described without the sonication step. Samples named ‘Wet’ included the humidification step during their preparation while ‘Dry’ samples did not. Results are summarized in Figure 2.

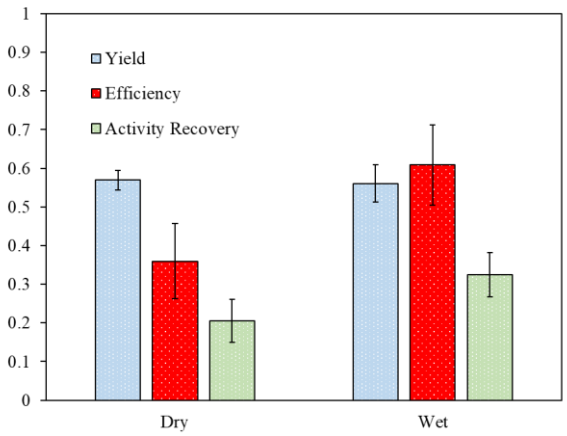

Figure 2. Humidity effect in CA-PIL Yield, Efficiency and Activity Recovery, defined in equations 1, 4 and 5, respectively. N≥3, error bars are standard error. 'Dry' (non-humidified sample) and 'Wet' (humidified sample) are compared

CA suspension hydration showed a positive impact on the efficiency (specific activity of immobilized CA/ specific activity of free CA) as more immobilized enzyme could exploit its catalytic activity. The yield (amount of CA immobilized/ amount of CA used in the formulation) was not significantly different for Wet and Dry while the activity recovery increased. Activity recovery gives a measure of the efficiency of the overall immobilization process accounting for enzyme losses during immobilized enzyme preparation and for enzyme activity reduction upon immobilization[94]. The activity recovery of the immobilized enzyme was also improved by the humidification step. As a control, the increase in humidity of the IL monomer when it was exposed to the humidified stream was investigated by Karl-Fischer titration. The dry IL presented a water content of 115 ppm (0.0115% $_{w/w}$). After the wetting treatment its humidity increased up to 5086 ppm (0.586% $_{w/w}$). Thus, we can state that the humidity of the sample was higher after the treatment. Free radical polymerizations are not sensitive to moisture[103]. Consequently, the IR spectra (see CA-PIL physicochemical characterization) showed that humidification did not have a significant impact in PIL chemical structure. We surmise that the higher efficiency and activity recovery values might be due to hydration of the suspended lyophilized enzyme and perhaps to limited enzyme solubilization in the hydrated IL monomer solution and not due to a variation on the resulting PIL chemical structure.

EFFECT OF CA PARTICLE SIZE

Following the analogy of the utilization of suspended enzymes in non-aqueous media biocatalysts, a reduction of enzyme particle size is expected to lead to a decrease in the mass transfer resistance as it is easier for the substrate to reach the enzyme active site[94,104,105]. To further improve the enzymatic activity, samples of wet CA suspensions were subjected to different sonication times with the objective of reducing the suspension particle size (15, 30, 45, 60 and 75 min, named Wet15', Wet30', Wet45', Wet60' and Wet1h15' respectively). As expected, sonication allowed an increase in yield compared to samples without sonication (Figure 3). By sonication, enzyme aggregates were broken down into smaller pieces improving entrapment and leading to a better homogenization and dispersion of the enzyme. This was confirmed by observation of the suspension using optical microscopy. As shown in the Figure 4, longer sonication times yielded smaller enzyme flakes. Furthermore, the increase in efficiency as the sonication time was increased from 15 to 30 min could be associated to the reduction of mass transfer limitations as the CA agglomerates size was reduced. Beyond 30 min, however, efficiency was progressively reduced. This could be explained by the detrimental effect that sonication can have on enzyme activity[104,106]. A tradeoff between enzyme deactivation and aggregates size reduction could explain this trend. Interesting, sonicated samples presented lower activity than samples without sonication. To provide more insights about the effect of enzyme particle size reduction, reusability experiments were carried out for two cases: wet sample without sonication (Wet) and wet sample with 30 min sonication (Wet30'). In both cases, there was a reduction in retained activity during the first cycle (Figure 5). The reaction rate after CA-PIL removal was considerably larger than the blank, which indicated that enzyme leaching could justify the significant decrease of enzyme activity during the first cycle. This decrease was much more pronounced in the case of samples without sonication (Wet). As expected, the sample subjected to sonication provides a better entrapment of the enzyme and better reusability. A better entrapment and homogenization is probably the cause of the activity reduction in sonicated samples compared to without sonication. Further analyses were done using samples that were treated with sonication during 30 min (Wet30').

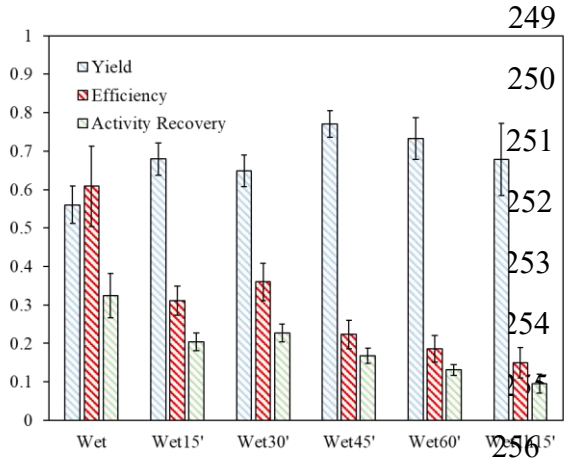

Figure 3. Sonication time effect in CA-PIL Yield, Efficiency and Activity Recovery, defined in equations 1, 4 and 5, respectively. N≥3, error bars are standard error. Samples that underwent sonication times of 15, 30, 45, 60 and 75 min are named Wet15', Wet30', Wet45', Wet60' and Wet1h15' respectively. Samples without sonication are named Wet.

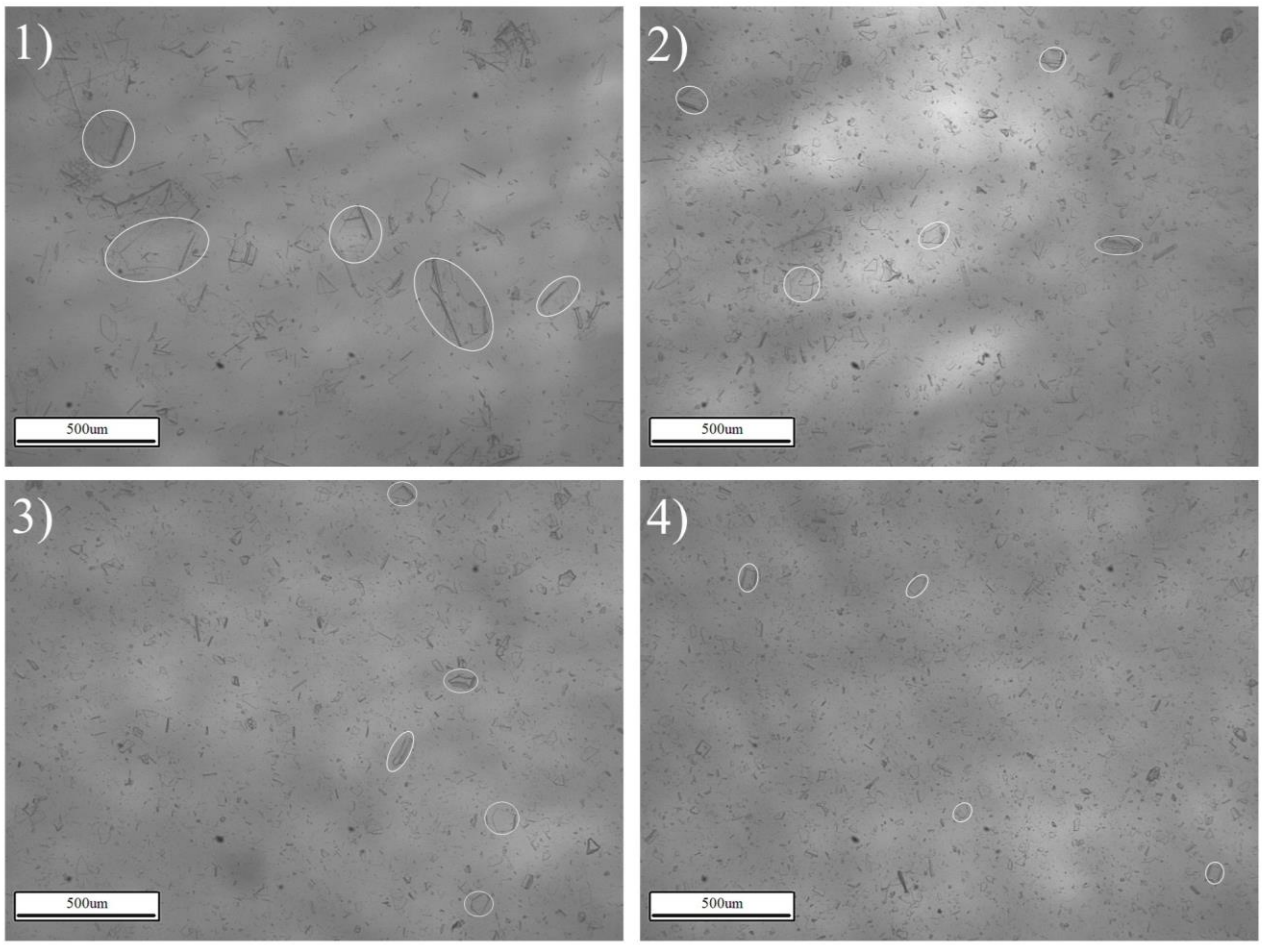

Figure 4. Effect of sonication time in the suspension enzyme particle size. 1) Without sonication and 2) -4) after 15, 30 and 75 min respectively. The biggest particles in each image are marked with white circles to help image comparison.

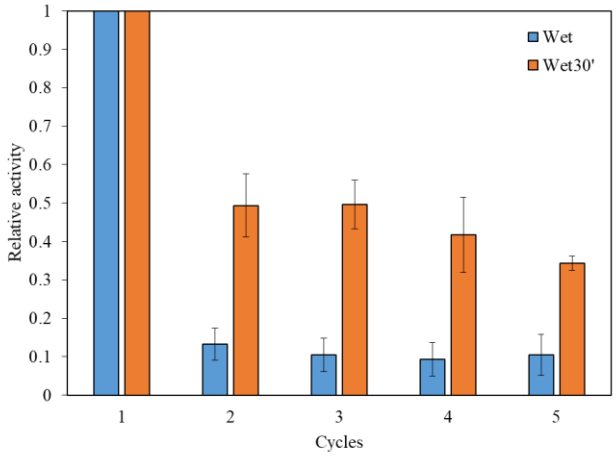

Figure 5. CA-PILs reusability expressed as Relative activity, defined in equation 7. N=3 for Wet30' and N=2 for Wet, error bars are standard error.

CA-PIL PHYSICOCHEMICAL CHARACTERIZATION

CA-PILs rubbery nature made the grinding process difficult. The films obtained after polymerization were therefore cut and teared apart forming smashed polymer pieces. In general, CA-PILs were in the form of thin flakes of about 20 µm thickness and 1-5 mm length, see Figure 6. No porosity was visible in SEM. Relatively large particle size and lack of porosity probably had a detrimental effect in the observed activity as the internal mass transfer was probably decreased. As shown in Figure 6 2) and 2 a), the top and cross-sections of PIL films were very smooth. In the other hand, for CA-PILs (Wet30') some structures were identified. The top surface of CA-PILs (Wet30') was mostly smooth with some isolated distinct shapes, see Figure 6 3). In the cross-section of CA-PILs (Wet30') abundant slits were present, see Figure 6 4). This is probably indicating that most of the entrapped enzyme was embedded in the interior of the PIL with little directly exposed to the surface.

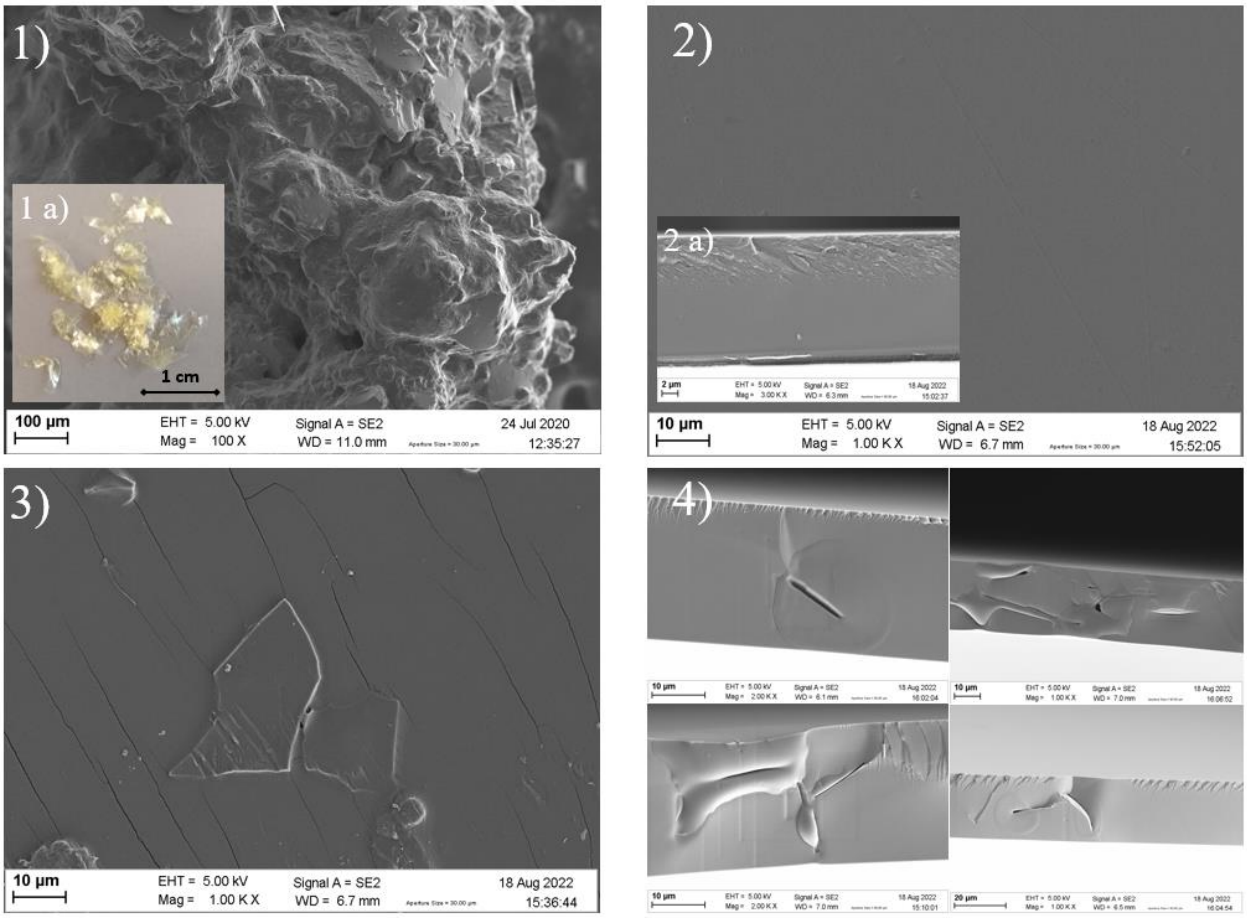

Figure 6. SEM images of materials developed in this work. 1) and 1 a) are a SEM image and a photography of grinded CA-PILs (Wet30') , 2) and 2 a) are the top and cross-section views of PIL films (no grinded), 3) and 4) are the top and cross-section views of CA-PILs (Wet30') films (no grinded), respectively.

To gain more insight about the presence of immobilized CA and its distribution, EDX experiments were performed. S, C, N and O signals belong to both polymer and enzyme (see Figure S7 in Supporting information). Since a Zn atom is at enzyme's core, it can be diagnostic. Several authors followed this approach to study the immobilized CA presence/distribution in different carriers[107–111]. For the materials studied in this work, however, Zn signal was not observed. Optical microscopy provided more conclusive results, see Figure 7. Poly(ionic liquid) films were completely transparent. Thus, lyophilized enzyme flakes embedded within the PIL were readily observable. Such structures were not present in PIL prepared without CA.

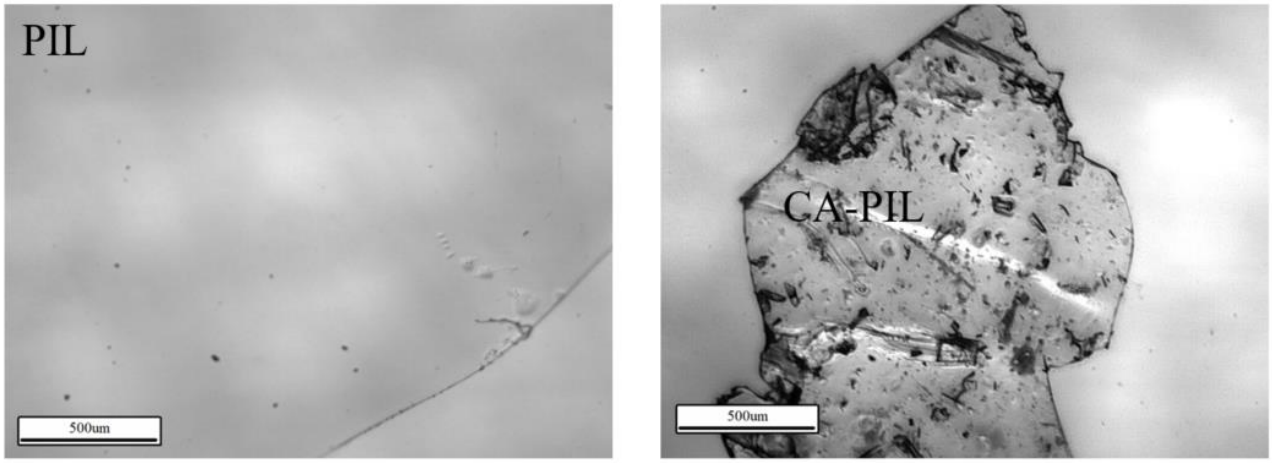

Figure 7. CA-PILs (Wet30') and PIL films observed using optical microscopy.

The chemical structure of IL monomer, PIL, Wet30' CA-PIL and CA were investigated by IR spectroscopy. Figure 8 shows the relevant spectra. The IL monomer and PIL spectra are similar to those reported in other works[112,113]. In the IL monomer spectrum, the signals at 1656 and 954-916 cm^{-1} correspond to the vinyl group in the imidazole cation[114,115]. The relative changes in the intensity of the 1658 cm^{-1} band were used to estimate the degree of polymerization. The band assigned to the imidazolium ring 654 cm^{-1}[1116] was utilized as internal standard peak[117,118]. The double bond conversion is close to 100% as the vinyl peak is not detectable in the spectra of polymerized samples indicating the success of the polymerization. CA signal corresponding to amide I (1635 cm^{-1}) was visible in the spectrum of CA-PIL Wet30' proving that the enzyme was present in the polymer.

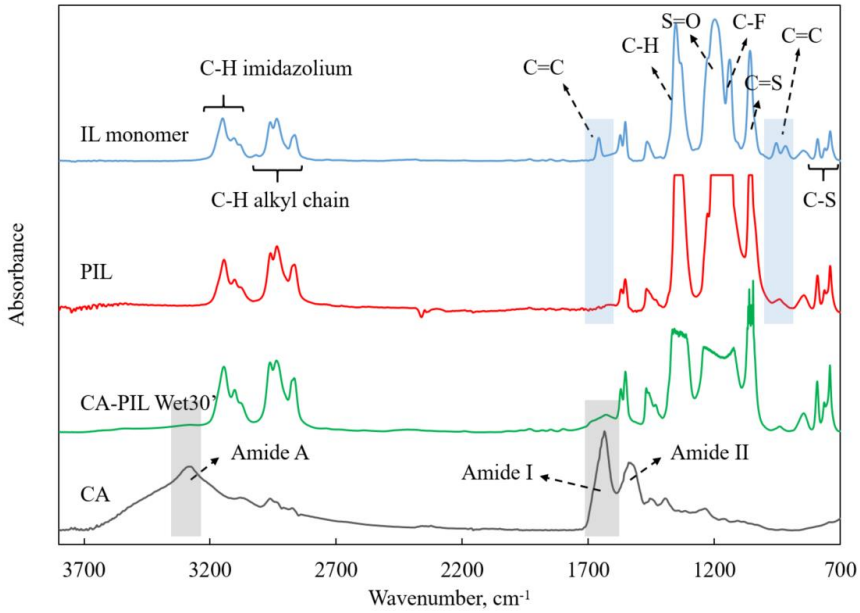

Figure 8. IR spectra of studied materials.

The TGA results were similar for IL, PIL and Wet30' CA-PIL samples. The curve for Wet30' CA-PIL is provided in Figure 9. There is a major drop of mass at about 400 °C which is consistent with the thermal degradation temperature range reported for poly([VEIm][NTf2])[119].

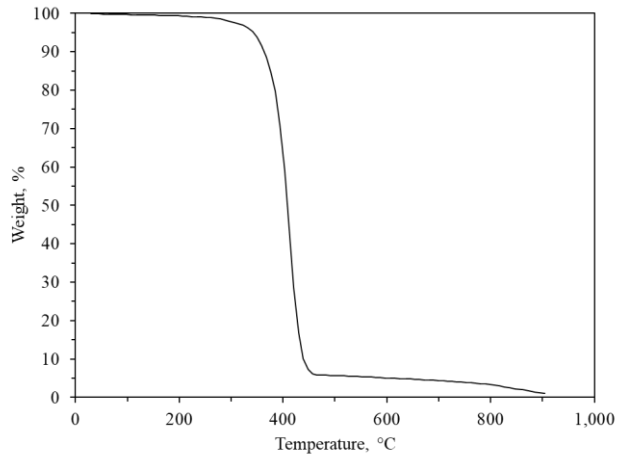

Figure 9. TGA results for CA-PILs.

KINETIC PARAMETERS

Kinetic parameters for p-NPA hydrolysis were determined to study the catalytic behavior of immobilized enzyme and to compare it to the free enzyme. kcat and Km were estimated using the Lineweaver–Burk plot. The data seems to

follow the Michaels-Menten kinetics, (Figure 10). For free enzyme kcat, Km, and kcat/Km values were 12.55 s^{-1}, 55.86 mM and 0.22 s^{-1} mM^{-1}, while for immobilized CA these were, 1.01 s^{-1}, 17.41 mM and 0.06 s^{-1} mM^{-1} respectively. Although there is certain variation in the kinetics parameters reported in the literature for free bovine origin CA, see table 1, the obtained kcat/Km for free enzyme is in the range reported in other works. kcat for free enzyme is larger than for immobilized CA. This is usually associated to conformational changes in the enzyme during immobilization and/or to mass transfer limitations within the carrier[93,120]. Regarding, Km, it is smaller for immobilized CA than for free enzyme. This is usually attributed to an enhancement in the enzyme-substrate affinity compared to the free state [121,122].

Table 1. Reported values for kinetic parameters from free bovine CA.

kcat, s^{-1}	Km, mM	kcat/Km $mM^{-1}s^{-1}$	Reference
3.02	6.18	0.488	[123,124]
6.97	11.7	0.595	[125]
-	-	0.504	[126]
4.32	9.54	0.453	[90]
2.27	6.09	0.372	[127]
-	18	-	[128]
49.51	56.67	0.874	[129]
-	-	0.700	[130]
-	9.38	-	[91]
2.02	12.2	0.166	[131]
-	-	0.960	[132]
-	-	0.266	[133]

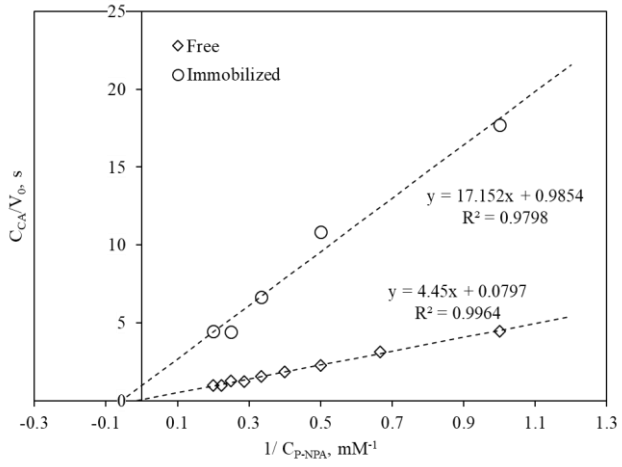

Figure 10. Lineweaver-Burk plot of free CA and CA-PILs. Experiments were performed at least in duplicate.

Nemestóthy et al.[134] reported strong deactivation (90-95%) of CA in the presence of the hydrophobic IL [BMIm][NTf2]. This IL is relatively similar to the monomer used in this study as they share the same anion. The anion structure plays an important role on the IL-enzymes interactions[79,135]. Their results contrast with the general view that enzymes are more stable in hydrophobic ILs because of the low interaction with them while enzymes suffer detrimental effects when they are dissolved in hydrophilic ILs[136–138]. To investigate the effect of IL monomer on the enzyme activity a series of activity experiments were performed. These are schematized in Figure 11. Three IL monomers were compared: [VHIm][Br], [VEIm][Br] and [VHIm][NTf2]. For [VHIm][NTf2], 2 mg of CA lyophilized powder was suspended in 1 ml of IL monomer and stirred for 30 minutes at room temperature. Then, CA was recovered using a filter and washed with abundant hexane to remove the IL. After drying, the recovered enzyme power was dissolved in 5 ml of Tris-HCl buffer and its activity was measured using the p-NPA assay and the enzyme concentration estimated by Bradford. An additional experiment was performed using only hexane as control. The measurements were performed in duplicate. The activity of the enzyme after exposure to the IL monomer was about 75% of a freshly prepared CA buffered solution. This decrease is larger than the control experiment using only hexane where the retained activity was 96%. Therefore, it can be concluded that interaction with the hydrophobic IL itself caused a slight reduction in the activity of the enzyme. On the other hand, when CA was dissolved in a 25% $_{w/w}$ buffered solution of [VHIm][Br], a hydrophilic IL monomer intermediate in the preparation of [VHIm][NTf2], and [VEIm][Br], a hydrophilic IL monomer with a shorter alkyl chain, the enzyme activity was completely lost for the first case and a retained activity of 38% was measured for the latter. This is in agreement with the general trend that enzymes are more

stable in hydrophobic ILs than in hydrophilic ones. Additional controls were executed to account for the interference of the ILs in the activity assay. The results shed light on the activity reduction observed after immobilization. The interaction with the IL monomer seems to cause a slight reduction of activity. However, this decrease is not sufficient to match the decrease observed after immobilization, therefore, additional phenomena must be contributing to the activity diminution. As indicated previously, the CA-PILs structure is dense which also supports internal mass transfer limitations as an additional cause of the reduced kcat value. This hypothesis is further backed up by the preliminary activity results introduced previously. They suggested that a certain enzyme loading was required to observe activity (see Figure S8). In line with the results of this work are those from Grabner et al.[49]. They prepared solid phase IL coated enzymes and found that increments in the coating thickness (by increasing the proportion of molten IL to enzyme) led to lower activities due to the mass transfer limitations caused by the coating.

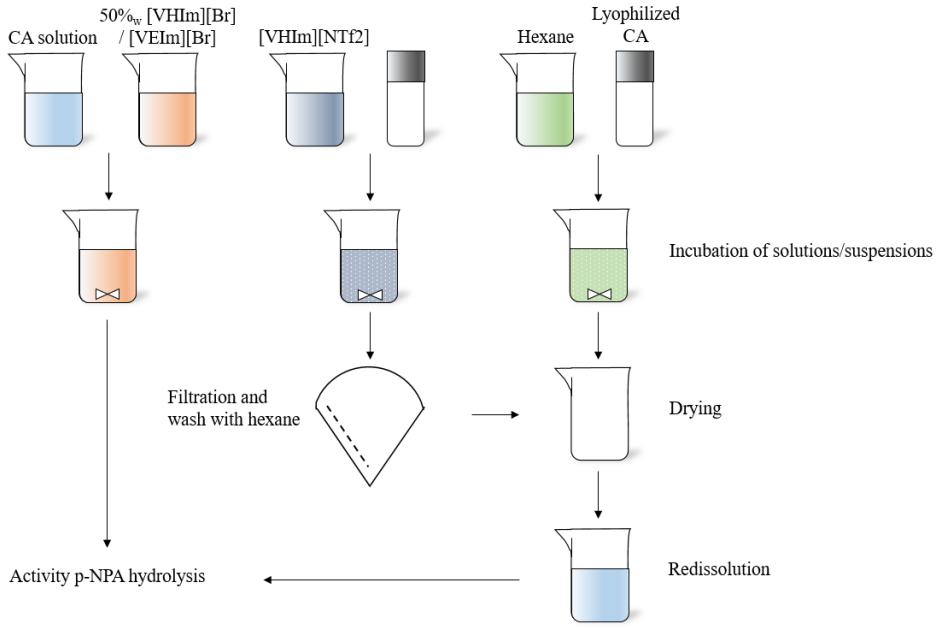

Figure 11. Experiments to compare the activity of CA after exposure to different ILs. Experiments were performed at least in duplicate.

THERMAL STABILITY

CA thermal stability was investigated between 20-70°C due to its interest in post-combustion CO_2 capture[4]. The activity of CA-PIL and free enzyme after been incubated at different temperatures is shown in Figure 12. Both curves presented the typical bell shape, with an optimal temperature around 40°C for the free enzyme and around 30 °C for

CA-PIL. Contrary to what is usually seen in enzyme immobilization[139], CA-PIL had lower thermal stability as its relative activity rapidly decreased above 30°C. Enzymes are complex structures held together by weak forces. Denaturation by heating is due to partial or complete unfolding of the protein structure[140]. Part of this process is reversible, while it becomes irreversible when enzymes start to agglomerate and form insoluble precipitates[140,141]. When immobilized, enzymes are usually stabilized by structural rigidification through multipoint covalent binding, steric hindrance by imprisonment and dispersion within the carrier which avoids aggregation[111,141,142]. Although the general trend is the stabilization of enzymes after immobilization[5], some authors have reported that immobilization can make the enzyme more susceptible to thermal denaturation. For example, bovine serum albumin secondary structures were less stable when adsorbed on graphene oxide according to far-UV CD measurements[143]. Similarly, laccases adsorbed on aluminium minerals presented lower relative activities than free enzyme after 1h incubation at 45°C[144]. The activity reduction was hypothetically attributed to changes in enzyme ternary structure. For CA enzyme, covalent immobilization in a poly(urethane) foam also reduced thermal stability[145]. The authors of the study highlighted that after incubating free enzyme to high temperature, the activity is partially recovered when the solution is cooled down due to protein partial refolding. The larger the incubation temperature, the lower the activity that can be recovered. They speculated that unfolded immobilized enzyme would interact with the carrier hindering the refolding process. Lower thermal stability was also reported in CA covalently attached to a polycationic polymer[146]. In the article, it was suggested that the interaction between the enzyme and the amino groups of the polymer might lead to negative effects. Finally, a histidine-tagged CA was adsorbed on Ni based MOFs. At higher temperatures, the immobilized enzyme showed lower relative activities due to leaching from the support at these conditions[147]. In the materials reported in this work, extensive enzyme leaching at higher incubation temperatures was not the reason for the observed activity reduction. As indicated previously, CA molecules were not dispersed within the material which could have had a detrimental effect in the stability[148]. Interaction with the hydrophobic PIL could also contribute to the destabilization of the enzyme at higher temperatures as many enzymes suffer from destabilization when interacting with hydrophobic surfaces [148,149]. However, these reasons are just hypothesis as in the previously discussed examples of lower thermal stability of immobilized enzymes, the enzymes were dispersed and immobilized on hydrophilic carriers.

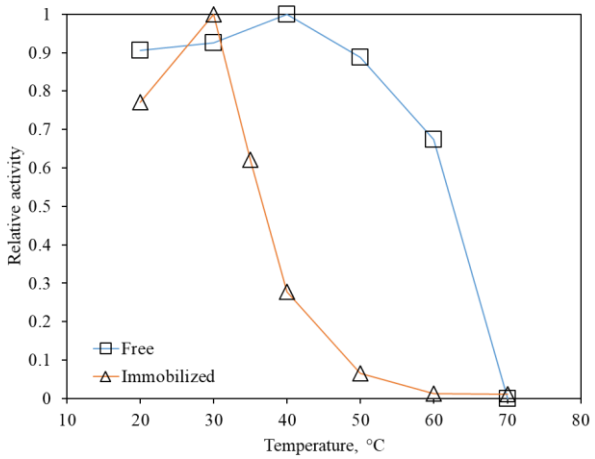

Figure 12. Thermal stability of free and immobilized CA (CA-PIL Wet30'). N=3, error bars are standard error.

CO_2 HYDRATION AND REUSABILITY

p-NPA hydration is often used to optimize and screen materials since it uses the same active center as the CO_2 hydration reaction. However, some authors have found discrepancies in the correlation of activities measured by both methods[150]. As a consequence, in order to evaluate a material, it is required to use CO_2 as substrate. Table 2 shows the specific WAU of immobilized and free enzyme. The CO_2 efficiency is lower in comparison to the p-NPA efficiency of immobilized enzyme. This is consistent with the results found in literature[109,111,151]. The specific activity of immobilized CA is lower than free enzyme probably due to mass transfer limitations within the, rather dense, PIL material. Moreover, CA-PILs could not be grinded easily into a powder to further reduce the particle size. Nevertheless, immobilization allows the easy reutilization of the support, see Figure 13. CA-PILs presented good reusability as the average relative CO_2 hydration activity remained above 85% during the first 4 cycles and larger than 60% for the fifth. Breakage of the support and the subsequent release of entrapped enzyme plus minor losses of CA-PIL during the cycles were confirmed to be contributors to this activity reduction. Control experiments were performed using PIL without enzyme. It was concluded that the PIL material did not present CO_2 hydration properties. The attempts to estimate the kinetic parameters of CO_2 hydration by a modified Wilbur and Anderson assay[152] did not provide conclusive results.

Table 2. CO_2 hydration activity for free and immobilized enzyme. N=3, variability reported as standard error.

	WAU/mg CA
Free CA	223 ± 13

CA-PIL Wet30’	12.9 ± 0.5

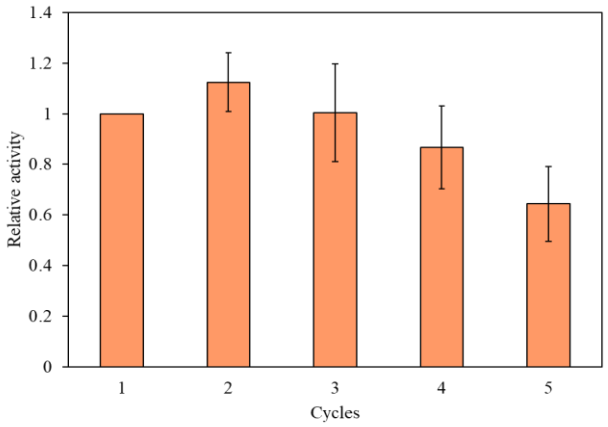

Figure 13. CA-PILs CO_2 hydration reusability expressed as Relative activity, defined in equation 7. N=3 for Wet30’. Error bars are standard error.

STORAGE STABILITY

In order to test CA-PIL Wet30’ stability, both immobilized and free enzyme were incubated in 0.1 M Na_2CO_3. As reported in Figure 12, both free and CA-PIL activities remained constant during 1 month. However, for free enzyme there was an initial trop of activity during the first week of incubation (~30% activity loss). As it was not the case for CA-PILs, it can be concluded that immobilization increased storage stability. The percentage of leached enzyme is similar in all samples regardless of the incubation time. Most CA that was not properly immobilized was lost during the first week.

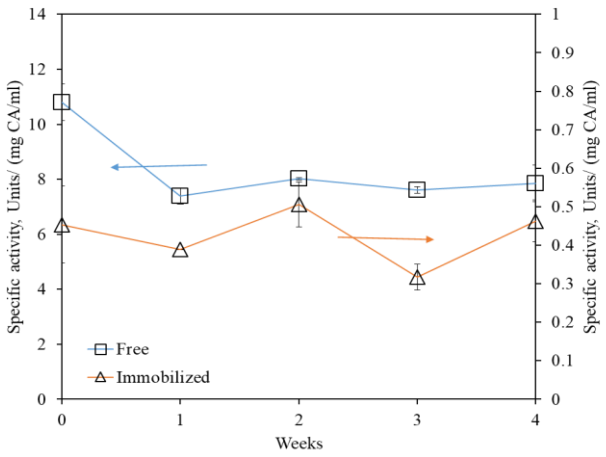

Figure 14. Free and immobilized CA storage stability in 0.1 M Na_2CO_3 at room temperature. N=3, error bars are standard error.

CONCLUSIONS

In the present study, carbonic anhydrase was immobilized in a polymerized ionic liquid. With the objective of maximizing enzyme activity and stability and CO_2 affinity, a hydrophobic ionic liquid was selected. As the enzyme was not soluble in the monomer solution, the enzyme lyophilized powder was suspended in the monomer solution and entrapped after subsequent polymerization. This represents a new approach of enzyme entrapment that could be interesting for enzyme immobilization in other types of substrates or polymers where the enzyme is not soluble in the carrier precursors solution. Despite being relatively low, the efficiencies displayed by carbonic anhydrase immobilized poly(ionic liquid)s are still comparable to those reported by some hydrophilic carriers[111,127,153,154]. The immobilization protocol was optimized in terms of efficiency. It was found that lyophilized enzyme suspension hydration improved catalytic efficiency. Reduction of suspension particle size by sonication also provided enhancements in immobilized enzyme activity and reusability. However, excessive sonication times presented a detrimental effect. The kinetics parameters for the p-NPA hydrolysis were computed for immobilized carbonic anhydrase and compared to free enzyme. Lower kcat/Km values were observed for the immobilized enzyme. Thermal and storage stability were also evaluated. Storage stability was improved with respect to free enzyme upon immobilization. However, the thermal stability was reduced. Immobilization allowed easy reutilization of the enzyme. Additionally, the biocatalytic materials developed in this work presented good levels of CO_2 hydration activity during five consecutive cycles making it an interesting material for accelerating the capture of CO_2.

SUPPORTING INFORMATION

A free of charge supporting information document is available containing:

- ^{1}HNMR and ^{1}CNMR, spectra of [VHIm][NTf2], [VHIm][Br] and [VEIm][Br].
- CA-PIL EDX spectrum.
- Effect of enzyme loading in activity
- Effect of UV light exposure in free enzyme activity

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Abstract graphic