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Immobilization of Carbonic Anhydrase for Biomimetic CO_2 Capture in a Slurry Absorber as Cross-Linked Enzyme Aggregates (CLEA)

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Novel post-combustion Carbon Capture and Storage (CCS) processes include absorption of CO_2 into aqueous solutions assisted by enzyme catalysis. Carbonic anhydrase EC 4.2.1.1 (CA) catalyzes CO_2 hydration and it has been proposed as industrial biocatalyst for biomimetic CCS processes. The present study reports on the use of bovine CA immobilized via cross-linking of enzyme aggregates (CLEA). The aim of this study was to improve the biocatalyst stability at the typical operating conditions of CCS processes (high temperature, alkaline pH, high salt concentration). The optimum conditions of the immobilization procedure were determined in terms of enzyme concentration and cross-linker concentration. In addition, a magnetic CLEA (m-CLEA) sample was prepared, based on cross-linking in presence of amino-functionalized paramagnetic nanoparticles. Immobilization yields was remarkable in both cases. No substantial differences were observed between conventional and magnetic CLEA. The use of magnetic CLEA enables effective separation of the biocatalyst from the reaction mixture and prevent drawbacks associated with CLEA aggregation and compaction induced by centrifugation and filtration.

1. Introduction

The reduction of CO_2 emissions from stationary sources – e.g. power plants fired with fossil fuels – is one of the targets of greenhouse gas control actions and research efforts to develop efficient Carbon Capture and Storage (CCS) (IPCC, 2005). Post-combustion CCS, consisting of carbon dioxide capture from flue gases after removal of particulates, NO_x and SO_x, is attractive as it can be used in retrofitting existing plants. Carbon dioxide absorption into aqueous solutions of alkanolamines is well-established, and currently represents the benchmark of CCS post-combustion treatments (Wang et al., 2011). It is based on regenerative absorption and concentration of CO_2 gaseous streams to be directed to geological storage. Drawbacks of the amine-based process are represented by amine oxidation and production of toxic volatile compounds, e.g. ammonia. Therefore, carbon dioxide absorption into alkanolamines based solvents requires additional processing to remove toxic products from both the aqueous and gaseous streams.
An alternative process has been recently proposed to replace the organic promoter (amines) with an environmentally friendly biocatalyst. The biomimetic process is based on the use of the enzyme carbonic anhydrase (E.C. 4.2.1.1) which effectively increases the CO_2 absorption rate in aqueous solutions (Russo et al., 2013). Carbonic anhydrase (CA) is a ubiquitous enzyme that catalyzes CO_2 hydration (Tripp eta al., 2001). Carbon dioxide absorbed into aqueous solution is chemically stabilized by hydration and/or hydroxylation. Hydration is catalyzed by CA and the reaction rate depends on both enzyme form and operating conditions. In particular, the turnover number depends on the class which CA belongs to. It may be as high as $1.40 \cdot 10^6$ s^{-1},

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the turnover number of the human isoform II (hCA II) (Vullo et al., 2013). Extensive CO_2 capture may be accomplished only if the absorption medium is an alkaline solution, characterized by satisfactory CO_2 absorption capacity. The design and optimization of biomimetic CCS processes require CA forms able to operate under conditions close to those adopted in the industrial processes. These concepts are fully reviewed by several authors (Lacroix and Larachi, 2008; Russo et al., 2013a). The main parameters affecting CA performance and durability include the presence of pollutants in the gas phase, temperature, pH and salt concentration. Pollutants usually contained in flue gases – fly ashes, NO_x, SO_2, mercury and chlorine – may affect enzyme activity. However, CO_2 capture is typically performed at the end of the post-combustion treatment, where the concentrations of these pollutants are below the emission limits. The temperature of the absorption unit typically ranges between 40 and 60 °C. The temperature of desorption unit is typically slightly above 100 °C, but it can be reduced if the process is carried out under vacuum (typically 0.3 bar) (Chen et al., 2007). Finally, potassium carbonate solutions – salt concentration ranging between 20 % and 30 %wt and pH about 10 – are alkaline media characterized by good absorption capacity and competitive desorption costs with respect to MEA solutions (Chen et al., 2007).
Enzyme stability at process conditions may be increased by covalent immobilization techniques such as enzyme attachment on solid supports or cross-linking of enzyme aggregates. Cross-linked Enzyme Aggregates (CLEA) are novel and versatile carrier-free immobilized biocatalysts (Sheldon, 2011). Through this technique, enzymes are first aggregated into super-molecular structures promoted by appropriate precipitating agents, typically inorganic salts or organic solvents. Eventually, aggregates are stabilized by chemical cross-linking with a bi-functional reagent, usually glutaraldehyde. The final preparation of CLEA yields pure protein with high specific enzyme activity thereby maximizing volumetric productivity and space-time yields. In addition, CLEA exhibit a significantly enhanced storage and operational stability, they have low production cost and no inert solids to dispose of as no expensive carrier is needed. An exhaustive description of the general protocol for CLEA preparation is reported by Shoevaart (2004); the techniques has been applied to numbers of enzyme (see Podrepšek et al., 2012). On the other hand, separation of CLEA from reaction media by centrifugation or filtration leads to aggregation phenomena that result in increased and poorly controlled particle size whose main consequences are mass-transfer limitations and uncertain biocatalyst performances (Takelar et al., 2012).
The present study reports on the preparation of CLEA using bovine CA as reference enzyme. The optimum conditions of the immobilization procedure were determined in terms of carbonic anhydrase and cross-linker concentrations providing the best activity and immobilization yields. In addition, a magnetic CLEA sample was prepared, carrying out the cross-linking reaction in presence of amino-functionalized paramagnetic nanoparticles. Magnetized CLEA can be easily separated from the reaction mixture by the application of a magnetic field, eliminating the need of centrifugation and filtration.

2. Materials and methods

Bovine CA was supplied by Sigma Aldrich® as lyophilized powder extracted from bovine erythrocytes (≥ 75 % wt). Paramagnetic nanoparticles used for magnetic CLEA preparation (fluidMAG-Amine, Chemicell) presented an outer aminosilane matrix and a magnetite core. They were supplied as aqueous dispersion and their average diameter was 100 nm. Other chemicals, supplied by Sigma Aldrich® were: bovine serum albumin BSA as lyophilized powder (≥ 96 % wt), 25 % vol glutaraldehyde grade I, tris(hydroxymethyl)aminomethane (≥99.9 % wt), sulfuric acid (95 – 98 %), phosphate buffer sulphate (PBS) salt and ammonium sulphate (≥99 % wt).

2.1 Preparation of CLEAs

The immobilization procedure, referring to Shoevaart et al. (2004), included the following steps:

- Precipitation: bovine CA solution (10 g/L) was added to $(NH_4)_2SO_4$ saturated solution. Precipitation was carried out for 30 min under stirring;
- Cross-linking: 25 % glutaraldehyde solution was added to reaction mixture up to the desired final concentration. Cross-linking was carried out for 3 h;
- CLEA were separated from reaction mixture by centrifugation, then washed with PBS (pH 7.4). Washing cycles were performed four times.

Each step was carried out at room temperature and using a blade stirrer at 920 rpm mixing speed. Finally, CLEAs were stored at 4 °C in PBS. Initial bovine CA concentrations in the precipitation solution were 1 and 2 g/L. Such concentration were obtained adding 1 ml of bovine CA stock solution (10 g/L) to 9 mL $(NH_4)_2SO_4$ saturated solution and 2 mL of bovine CA stock solution (10 g/L) to 8 mL $(NH_4)_2SO_4$ saturated solution. These ratios were fixed according to the maximum bovine CA concentration (10 g/L) suggested by the supplier for

the stock solution. Glutaraldehyde concentrations in the cross-linking step were ranged between 50 and 150 mM.
Preparation of magnetic CLEA followed the same procedure, but 0.2 mL of nanoparticles suspension (5 mg) were added to the enzyme solution before precipitation. Moreover, CLEA separation was performed under a magnetic field instead of centrifugation. Figure 1 shows a schematic representation of conventional and magnetic CLEA.

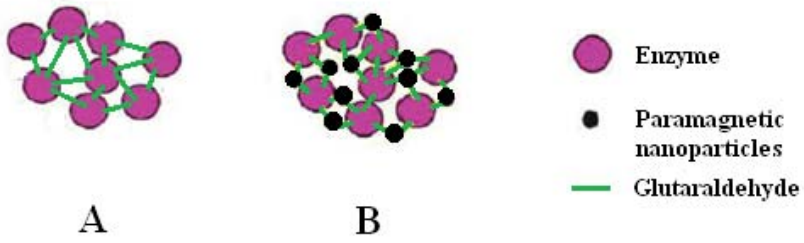

Figure 1: Schematic representation of (A) conventional and (B) magnetic CLEA.

Total protein concentration was assessed by monitoring the optical absorbance of aqueous solutions at 280 nm before and after precipitation. The protein content of the recovered fractions of the washing buffer was also assayed. The total amount of precipitated enzyme was calculated as the difference between the total initial loading of carbonic anhydrase and the residual carbonic anhydrase in the liquid after precipitation and in the washing buffer. The immobilization yield was calculated as the ratio between the total amount of precipitated CA and the initial amount of CA dissolved in liquid phase.

2.2 Activity assay

Activity assay of bovine CA CLEA was performed following the titrimetric method reported by Worthington (1993) for free CA and adapted to the CLEA suspension, as reported, in a previous work, on immobilized CA on solid support (Russo et al., 2013b). CA activity was measured as the rate of hydration reaction of carbon dioxide dissolved in 2 mL of a saturated aqueous solution added to 3 mL of 20 mM tris(hydroxymethyl)aminomethane sulfate buffer (TRIS sulfate) at 0°C. In particular, the time elapsed during pH decay from 8.3 to 6.3 was measured. CLEA concentration was properly tuned so that the measured time was longer than 20 s. The activity *A* was expressed as Wilbur-Anderson units per unit volume of incubated CLEA suspension (WAU/mL) according to Eq(1) (Worthington, 1988):

$$A = \frac{t_{blank} - t_{AC}}{t_{CA}} \frac{df}{v_{CA}} \quad (1)$$

where t_{blank} and t_{CA} are hydration times measured during tests carried out in absence and presence of CA, respectively; d_f is the dilution factor of the CLEA sample, and v_{CA} the added volume of the CLEA suspension (0.05 – 0.1 mL). Assays were repeated at least three times.
According to Schoevaart et al. (2004) the activity of each CLEA sample was calculated as the difference between the activity of the suspension at the end of the cross-linking stage and the activity of the liquid supernatant recovered after CLEA separation.

3. Results

Immobilization tests were performed in order to assess the optimum formulation conditions in terms of initial bovine CA and cross-linker concentration. In particular, initial bovine CA concentrations of 1 and 2 g/L and glutaraldehyde concentrations ranging between 50 and 150 mM were used. Precipitation yields of about 80 and 90% were observed at initial bovine CA concentrations of 1 and 2 g/L.
The specific activity of CLEA is reported in Figure 2 as a function of the initial concentration of bovine CA and glutaraldehyde. The activity did not change substantially by changing the ratio between bovine CA and glutaraldehyde concentrations. An average value of about 4.8 ± 0.6 WAU/mg_{CA} was observed for the entire set of samples, with the exception of the sample prepared with 1 g/L bovine CA and 50 mM glutaraldehyde. In that case a larger activity of about 68 ± 16 WAU/mg_{CA} was observed. Moreover, the activity of the liquid supernatant after the first washing in PBS was fairly large (34 ± 14 WAU/mL) compared with that assayed on

the other samples (almost null). These findings suggest that preparation of CLEA with 1 g/L bovine CA and 50 mM glutaraldehyde solutions resulted in poor cross-linking that resulted into extensive release of free bovine CA when CLEA suspensions were first diluted in the assay buffer. This effect was not observed in the sample prepared with the same ratio between bovine CA and glutaraldehyde with 2g/L bovine CA and 100 mM glutaraldehyde, possibly because of the different concentration of glutaraldehyde. Glutaraldehyde may be rapidly consumed by the free bovine CA residue of precipitation step, so 50 mM may be an insufficient concentration of cross-linker in the presence of about 0.2 g/L free bovine CA. In the case of 2 g/L bovine CA and 100 mM glutaraldehyde, the cross-linker concentration may be large enough to react with both residual free and precipitated bovine CA.

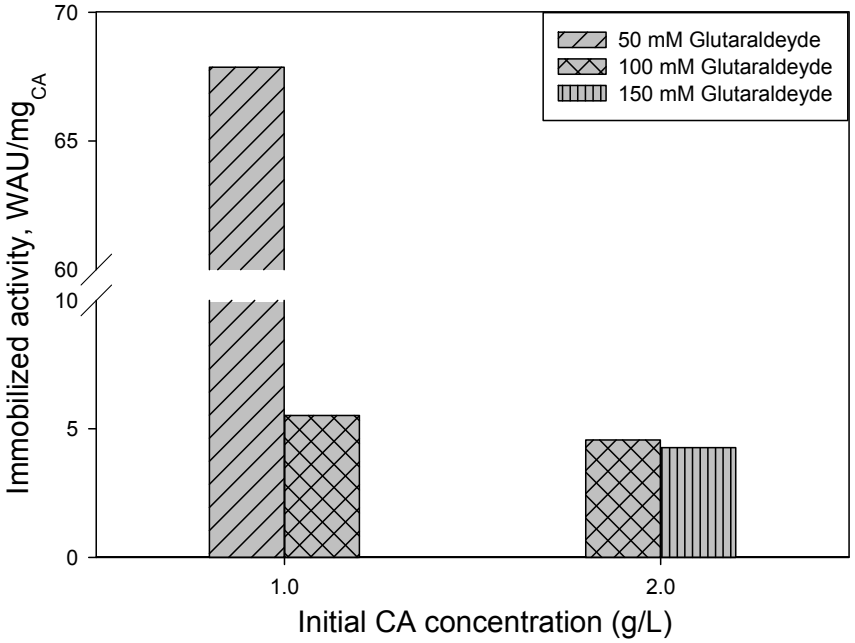

Figure 2: CLEA specific activity (per unit mass of immobilized CA) as function of initial CA and glutaraldehyde concentrations.

Since no change in activity was observed for the largest cross-linker concentration, preparation of CLEA at 2 g/L of bovine CA and 100 mM glutaraldehyde was considered as the best among those investigated. Accordingly, the CLEA functionalized with paramagnetic nanoparticles (m-CLEA) was prepared at these optimal conditions. Immobilization yield and specific activity of m-CLEA are reported in Table 1 and compared with those of the non-magnetic CLEA prepared in the same conditions. It is remarkable that immobilization yield and specific activity are not affected by inclusion of paramagnetic nanoparticles.

The beneficial effect of magnetic separation was assessed through activity assay on m-CLEA after washing and magnetic field separation. The activity assessed on the m-CLEA after magnetic field separation and washing was nearly the same as the activity assessed on the whole CLEA suspension after cross-linking (3.0±0.2 WAU/mg$_{CA}$). On the contrary, activity of centrifuged and subsequently washed CLEAs was lower (1.4±0.4 WAU/mg$_{CA}$) than that assayed with the whole CLEA suspension. It is inferred that centrifugation and filtration of CLEA may induce morphological modifications (e.g. increase of cluster size and/or compaction) that bring about apparent loss of enzyme activity.

Table 1: Results of immobilization yield, CLEA concentration and immobilized activity of bovine CA CLEA and bovine CA m-CLEA prepared at 2 g/L bovine CA and 100 mM glutaraldehyde.

Sample	Immobilization yield (%)	CLEA concentration (mg/mL)	Specific activity (WAU/mg$_{CA}$)
CLEA	89.6±2.6	1.65±0.05	4.6±0.5
m-CLEA	91.3±1.4	1.8±0.03	4.0±0.1

4. Conclusions

The present investigation, framed in a broader study on biomimetic CO_2 capture, has addressed the preparation of carbonic anhydrase cross-linked enzyme aggregates - CLEA - with a clue on the potential of using magnetized CLEA to facilitate separation and re-use of the biocatalyst. Aggregates were prepared following the procedure developed by Shoevaart et al. (2004). The effect of bovine CA and glutaraldehyde concentration on immobilization yields and specific activity of the biocatalyst have been characterized. Immobilization yields of 80 – 90 % were obtained under all the formulation conditions, as expected.
Specific activity of bovine CA CLEA was in the order of 4 WAU/mg_{CA}, and was barely affected by glutaraldehyde concentration and by the immobilization yield.
These results can be compared with those related with bovine CA immobilization on commercial aldehyde activated micro-beads (Peirce et al., 2014). The preparation of CLEA provided remarkable improvement of immobilization yield with respect to that obtained with covalent attachment on micro-beads (90 % instead of 50%). On the other hand, bovine CA CLEA showed a specific activity (4 WAU/mg_{CA}) smaller than that assessed for bovine CA immobilized on micro-beads (17 WAU/mg_{CA}). The observed reduced activity can be due to several reasons. Bovine CA may deactivate in the presence of glutaraldehyde concentrations typically adopted for CLEA preparation (Shoevaart et al., 2004) as a consequence of the large number of reactions involving the bi-functional reagent and the enzyme residues (Barbosa et al., 2014). Moreover, because the morphology of the heterogeneous biocatalysts (CLEA and CA immobilized on micro-beads) strongly influences their activity, the smaller activity of CA CLEA may be related to a larger average size of CLEA clusters with respect to those of CA immobilized on micro-beads.
Concerning the optimization of cross-linker concentration, glutaraldehyde concentration smaller than 100 mM are insufficient to provide complete cross-linking of precipitated bovine CA aggregates, as free enzyme may be leached in aqueous solution.
Noteworthy, the specific activity of magnetized CLEA was only slightly smaller than that of non-magnetic CLEA, prepared under the same formulation conditions. This finding confirms that the presence of paramagnetic nanoparticles does not interfere significantly with enzyme cross-linking and activity.
The potential of magnetized aggregates is better appreciated when considering the activity of CLEA upon recovery and re-use. A pronounced activity drop is observed once CLEA are recovered from the suspension by centrifugation and filtration. On the contrary, recovery of magnetized CLEA by magnetic separation does not bring about any significant drop in enzyme activity. This result encourages further testing of the potential of magnetic separation at pilot and industrial scales as a tool to enhance durability and re-usability of the immobilized biocatalyst.

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