ORIGINAL RESEARCH

Di Si et al

Combining Active Carbonic Anhydrase with Nanogels: Enzyme Protection and Zinc Sensing

Supporting Information

### Reagents

Carbonic Anhydrase from bovine erythrocytes, acrylamide, GDMA (glycerol dimethacrylate), AOT (Sodium dioctyl sulfosuccinate), Brij 30 (polyoxyethylene (4) lauryl ether), Tween 80 (polysorbate 80), Span 80 (sorbitan monooleate), PEG 400 (Polyethylene glycol 400), tergitolTM NP-9 (Nonylphenol Ethoxylates), ammonium persulfate, TEMED (N,N,N’,N’-tetraethylmethylenediamine), MOPS (4-morpholinepropanesulfonic acid), p-nitrophennyl acetate, Acetazolamide, bicine (N,N-Bis(2-hydroxyethyl) glycine), NTA (nitrilotriacetic acid), DPA (2,6 pyridine dicarboxylic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). APMA (N-(3-aminopropyl) methacrylamide hydrochloride) was purchased from Polyscience (Warrington, PA, USA). Dapoxyl sulfonyl chloride was purchased from Invitrogen (Eugene, OR, USA). Sulfo-SMCC (Sulfosuccinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate) was purchased from Soltec Ventures (Beverly, MA). Chelex 100 resin was purchased from Bio-Rad (Hercules, CA, USA). Coomassie Plus (Bradford) Assay Reagent was purchased from Thermo Fisher Scientific (Pierce) (Rockford, IL, USA). All buffer solutions were prepared using 18MΩ Milli-Q water purified by a Millipore Advantage A10 system and passed through a Chelex 100 column.

### Synthesis of Dapoxyl Sulfonamide

Dapoxyl sulfonamide is not commercially available; therefore, it was synthesized from the commercially available precursor Dapoxyl sulfonyl chloride (DSC). [1] DSC also reacts with water and the side reaction produces a fluorescent by-product, Dapoxyl sulfonyl acid. To reduce the side reaction, a 0.5 M ammonia solution in 1,4-dioxane was used instead of ammonium hydroxide. A 600 µl ammonia dioxane solution was added into a small vial containing 10 mg DSC and the reaction mixture was stirred at ice-cold temperature for 2 hours, in the dark, for the reaction to complete. Then the dioxane was removed by rotary evaporation.

The product was dissolved in 200 µl THF (Tetrahydrofuran), and 2-3 ml water was added. Then the pH of the solution was adjusted to about 5 by adding one drop of 1 M HCl. After sitting at room temperature for about 30 minutes, the DPS precipitated, leaving the Dapoxyl sulfonyl acid in solution. The mixture was then centrifuged at 5000 rpm for 10 minutes and the supernatant was discarded. This procedure was repeated three times and the precipitate was freeze-dried. The product was further purified by an Al2O3 column containing about 50 g Al2O3. The column was first run with 80 ml CH2Cl2, eluting out Dapoxyl sulfonyl acid, and then the mobile phase was changed to methanol (80 ml), eluting out DPS. The elution was collected, and the solvent was rotary evaporated to obtain a dry yellow powder.

Product purity was assessed by Al2O3 TLC plates. DPS was identified by its yellow fluorescence when excited by a hand-held UV light while the Dapoxyl sulfonyl acid exhibits a much weaker, blue fluorescence. An analysis of the product was also performed on a Micromass LCT Time-of-Flight mass spectrometer with Electrospray and APCI and the purity was estimated to be 90 % (m/Z of DPS is 344). The concentration of DPS was determined by its absorbance at 365 nm (ε = 22,000 M-1cm-1). [1]

### The interference of surfactants on sulfonamide fluorescence

Solutions of 1% w/v Brij 30, Tween 80, Span 80, Triton X-100, AOT, 1-Hexanol and PEG 400 were prepared in 10 mM MOPS. Solutions of 5 µM sulfonamides were also prepared in 10 mM MOPS. A 2 ml 5 µM sulfonamide solution was transferred into a cuvette, and the fluorescence of DPS (excitation 365 nm, emission 430-700 nm) or Dansylamide (excitation 330 nm, emission 400-650 nm, see Table S-1) was recorded on the fluorometer. Then, 20 µl of each surfactant solution was added to the sulfonamide solution, and the fluorescence changes induced by the surfactants were measured at 535 nm for DPS and at 450 nm for Dansylamide, respectively.

### Removal of Zn2+ from CA and nanogels

The preparation of apo CA has been described in the literature. [2] Basically, for free enzyme, a 20-50 µM CA solution was first prepared by dissolving CA in 10 mM MOPS (pH 7.2) containing 25 mM 2,6 pyridine dicarboxylic acid (DPA). This solution was stored at 4°C overnight, and the excess DPA was removed by serial dilution with 10 mM MOPS (pH 7.2). This was followed by concentration in an Amicon Ultra Centrifugal Filter Unit with a 10 kDa membrane cut-off size. The apo-enzyme was then further purified by running it through a 10 cm Sephadex G-25 column. For any experiments involving apo CA, all reagents were of the highest purity available. To minimize metal contamination, all solutions were made using 18 MΩ Milli-Q water treated with Chelex 100 resin, and all the plasticware was pre-treated with 18 MΩ Milli-Q Chelex 100-treated water. The apo CA concentration was determined by a *p*NPA assay after addition of excess Zn2+.

Regarding the nanogel, a 25 mg/ml nanogel suspension containing CA was prepared in 10 mM MOPS (pH 7.2) and 25 mM DPA, and the suspension was kept at 4°C overnight. The particles were washed with 50~60 ml 10 mM MOPS in a pre-treated low metal Amicon ultra-filtration cell to eliminate excess DPA.

### SEM imaging

Nanogels were dispersed in water at a concentration of 0.1 mg/ml, and the sample was sonicated for 30–60 minutes to prevent aggregation of the particles. A drop of the nanogel suspension was placed on a metal grid coated with carbon film and then dried gradually at room temperature. The sample was sputter coated with gold and visualized with a Philips XL30FEG scanning electron microscope.

**Table S-1. Dansylamide as a fluorescent binding ligand of CA** [3]

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | λEX (nm) | λEM (nm) | Quantum Yield | Kd (µM) |
| Dansylamide | Free | 330 | 560 | 0.08 | N/A |
| Bound | 330 | 450 | 0.55 | 0.8 |

λEX and λEM are the Excitation and the Emission wavelengths of Dansylamide, and Kd is the binding affinity of Dansylamide to CA.

**Figure S-1.** (a) Adjusted slope versus the concentration of CA. This calibration curve of the enzyme activity assay (pNPA assay) was used for quantifying the active CA inside the nanogels. (b). The pNPA assay of CA encapsulated AOT/Brij-30 nanogels (sample A in Fig 3) (red) and blank nanogels (blue).

**Figure S-2.** Coomassie Blue protein assay. (a) Coomassie Blue binds with both active and deactivated CA with an absorbance at 595 nm. Therefore, this assay was used for quantifying the total enzyme encapsulated in the CA-encapsulated nanogels (CA-enc-NG) and blank nanogels (Blank NG). (b) AOT and Brij 30 also interact with Coomassie Blue and increase its absorbance at 595 nm in a similar way as CA. For this reason, blank nanogels prepared under the same condition as the CA encapsulated nanogels were tested and used as a background. By subtracting the background caused by the residual surfactants in the blank nanogels, the estimation of the total protein amount in CA encapsulated nanogels became more accurate. (c) The absorbance of Coomassie Blue versus the concentration of CA. This calibration curve of the Coomassie Blue protein assay was used for quantifying the total CA inside the nanogels.

**Figure S-3.** The fluorescence interference of the sulfonamide fluorophores, Dapoxyl Sulfonamide (DPS) and Dansylamide (DNSA), by the surfactants. Controls are 5 µM DPS/DNSA in 10 mM MOPS buffer, while the final concentrations of surfactants are 0.01% w/v. All non-ionic surfactants used in the nanogel synthesis in this study (Brij 30, Tween 80 and Span 80) enhance the fluorescence of DPS and DNSA. Excitation: DPS 365 nm, DNSA 330 nm; emission DPS 535 nm, DNSA 450 nm.

**Figure S-4.** Free Zn2+ sensing using the CA encapsulated nanogels (sample D in Figure 2). This graph shows no fluorescence change as Zn2+ is added. The experiment was performed in 10 mM MOPS (pH 7.2) containing 10 mM NTA at 25°C.

### References

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