Supplementary Material (ESI) for Chemical Communications

SUPPORTING INFORMATION

Fluorescence characterization of immobilization induced enzyme aggregation

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MATERIALS

Medium molecular weight (MMW) chitosan and butyraldehyde were purchased from Sigma Aldrich (#538191-1L and #448877 respectively, Sigma-Aldrich St. Louis, MO). Cytoplasmic malate dehydrogenase (cMDH) was obtained from Sigma-Aldrich (M7383). The enzyme was dialyzed against phosphate buffer (pH 7, 50mM) to remove any pre-mixed ammonium sulfate added for enzyme stabilization during shipment. Glacial acetic acid, tris(hydroxymethyl)aminomethane (TRIS), HPLC grade methanol, and sodium hydroxide were purchased from Sigma-Aldrich and used without further modification. *AlexaFluor® 555* carboxylic acid succinimidyl ester and *AlexaFluor® 647* carboxylic acid succinimidyl ester were purchased from Molecular Probes (A20002 and A20006 respectively, Invitrogen Corporation, Carlsbad, California).

EXPERIMENTAL

Polymer preparation

All of the chitosan polymer used was first deacetylated to 95% according to published methods[1]. Specifically MMW chitosan was suspended in 45 wt% (11.25 M) NaOH and placed in an autoclave for 20 min at 121ºC. The resultant powder was then washed with 18MΩ-cm deionized water and followed by phosphate buffer (pH 7, 0.1M) before being dried *in vacuo* at 40 ºC. The MMW chitosan was then purified of residual fluorescent contaminants by rinsing 1 gram

aliquots over vacuum filtration with 500mL of 0.5M NaOH followed by 500mL of HPLC grade methanol. The powder was then dried at 40°C *in vacuo* (25mbar) for 24hrs. Butyl-modified chitosan (C4-chitosan) was prepared as described previously[2]. A portion of each polymer was then suspended in 0.2M acetic acid to create a 1 wt% solution and vortexed for 1 h in the presence of 2 and 5 mm diameter yttria stabilized zirconia oxide balls (Norstone, Wyncote, PA).

Enzyme labeling

Cytoplasmic MDH (cMDH) was dispersed in pH 8 sodium phosphate buffer (50mM) and combined with a stock *AlexaFluor® 555/647* solution (dissolved in DMSO) to a final 10:1 fluorophore to enzyme molar ratio. The mixture was then incubated at 4ºC for 24 hours under continuous stirring before being dialyzed against 1 L of 50mM TRIS (pH7.4) buffer for 12h followed by four subsequent 500mL volumes for 2.5hrs each in order to separate free probe from labeled enzyme. The final fluorophore to enzyme molar ratio was estimated to be 1:5, on average, for both *AlexaFluor® 555* and *647* by measuring the absorbance of the solution as a function of wavelength and then calculating the concentration of the protein and the probe using their respective molar extinction coefficients ($\varepsilon.c.$ = 66,510 cm^{-1} M^{-1} for cMDH and 112,000 cm^{-1} M^{-1} for *AlexaFluor® 555* and 270,000 cm^{-1} M^{-1} for *AlexaFluor® 647*). To confirm probe-protein binding, the steady state polarization was measured using an ISS PC1 spectrofluorometer (ISS, Inc., Champaign, IL). The polarization of *AlexaFluor® 555* and *AlexaFluor® 647* attached to the cMDH was measured at 0.35 and 0.38 respectively, and a significant increase when compared to the polarization associated with the probes free in solution, 0.02 and 0.02 respectively, indicating that the probe bound to a significantly larger rotating body, i.e. a protein[3].

Scaffold preparation

To each of the chitosan solutions stock solutions of 1:1 *AlexaFluor® 555:647* tagged cMDH was added to bring the final concentration of cMDH to 30μM in 0.5wt% solutions of chitosan, butyl or ALA-CHIT polymer. 50 μL of each solution described above was deposited onto a glass slide by drop casting from a micropipette. The slides were then frozen at -20 °C for 1hr. Thereafter, the slides were vacuum freeze dried for 12hrs. Although it is likely that some loss of activity

will occur during the drying process, this loss of activity should be consistent across all experiments (as the drying procedure is consistently applied).

Fluorescence Emission Spectra

For the FRET experiments solutions of 30μM *AlexaFluor®555-cMDH*, *AlexaFluor®647–cMDH* and their mixture (1:1 volume ratio) were first prepared in 50mM TRIS buffer (pH 7.4). Cross-linked mixtures of *AlexaFluor®555–cMDH* and *AlexaFluor®647–cMDH* (~ 1:1 volume ratio at 30μM for each pair) were realized by addition of glutaraldehyde to a final concentration of 600μM. Non-cross-linked solutions of *AlexaFluor®555*–cMDH and either native chitosan or C4-chitosan were realized by mixing the two respective solutions to a final concentration of 30μM protein and 20μM polymer (i.e. 1:1 volume ratio 60 μM labeled protein with 40 μM polymer solution). Non-cross-linked solutions of *AlexaFluor®647*–cMDH and either native chitosan or C4-chitosan were realized by mixing the two respective solutions to a final concentration of 30μM protein and 20μM polymer (i.e. 1:1 volume ratio of 60 μM labeled protein with 40 μM polymer solution). Non cross-linked mixtures of *AlexaFluor®555*–cMDH and *AlexaFluor®647*–cMDH in either native chitosan or C4-chitosan were realized by mixing both enzyme solutions with either polymer solution (1:1:2 volume ratio of 60 μM labeled proteins with 40 μM polymer solution).

For all emission scans, the fluorophores in solution were excited at 545nm and their fluorescence emission measured from 560nm to 700nm in a quartz cuvette using an ISS PC1 spectrofluorometer. A series of emission profiles were collected in 300s intervals over the span of 2.5hrs. The series of emission profiles for each solution were then corrected as per ISS's instruction for the light path of our specific instrument and stored for analysis.

The fluorescence emission spectra of the enzyme immobilized within the chitosan scaffold was measured by mounting a scaffold coated slide on to a variable-angle front surface sample holder (ISS, Champaign, IL) set at an angle of 22.5° to minimize scattered excitation light. The techniques for making the thin film scaffolds and their rehydration in enzyme buffer have been described previously[4]. Specifically, the scaffold films were re-hydrated with a 100μL drop of TRIS buffer (50mM, pH 7.4) and then sealed under a cover slip with wax. For all emission

scans, the fluorophores were excited at 545nm and their fluorescence emission measured from 560nm to 700nm. Each scaffold was scanned over the span of the same 2.5h and the emission profile was stored after it had been corrected for the optical filters and slits used in the experimental set-up.

SDS PAGE gels

The measurement of the protein molecular weight was performed on a graduated Criterion® TRIS-HCl gel (4 – 20%). The experimental protein samples were diluted in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol and 0.01% Bromophenol Blue) in a 1:3 volume ratio as recommended by the manufacturer. The protein ladder used for comparison was prepared in an identical manner, with the addition of 5% 2-mercaptoethanol to facilitate protein separation. To avoid returning the experimental samples to its original monomer state, 5% 2-mercaptoethanol was not added these protein samples. The gel was biased at 200V and run from 90 – 55mA with a run time of 55min. Afterwards, the gel was washed per the manufacturer's recommendation and stained with Imperial protein stain for 1hr. The gel was then rinsed over night in 18MΩ-cm de-ionized water at low agitation and photographed for analysis.

Light Scattering

The amount of light scattered from the sample was measured using the ISS spectrofluorometer. Specifically, the sample was excited at 340nm and the intensity of the scattered light (90° angle from the path of excitation) was collected at the same wavelength. To test for aggregation, the emission intensity was measured in 10s increments whilst 50μL of solution was removed every 15min for separation using a size exclusion (SDS PAGE) gel. The degree of aggregation (as measured relative to standard bands of know protein molecular weights) was then correlated to the measured light scatter.

FRET calculations

Baseline emission intensities as a function of wavelength (560 – 700nm) were measured solutions of 30μM *AlexaFluor®555–cMDH*, and 1:1 mixtures of 30μM *AlexaFluor®555–cMDH* and 30μM *AlexaFluor®647–cMDH* using an ISS spectrofluorometer. Also, the emission intensity as a function of wavelength was measured in the ISS for an identical 1:1 mixture of

AlexaFluor®555–cMDH and *AlexaFluor®647–cMDH (each* 30μM in 50mM TRIS buffer (pH 7.4)). in the presence of 150μM glutaraldehyde. The solution was repeatedly scanned (every 5min) over a period 2.5 h to record the FRET response to the increased aggregation states (identical to the protein and glutaraldehyde concentrations in the light scattering experiment). Each of the baseline measurements of *AlexaFluor®555–cMDH, AlexaFluor®647–cMDH,* and *AlexaFluor®555/647–cMDH* detailed were repeated in 20 μM deacetylated chitosan and 20μM C4-chitosan solutions. The emission intensity of each solution was scanned 10 times and the resultant radial separation average (Eq. 5) and standard deviation are reported as bars. 20 μL of each solution described above was deposited onto a glass slide by drop casting from a micropipette. The slides were then frozen at -20 °C for 1hr. Thereafter, the slides were vacuum freeze dried for 12h. Each scaffold was then scanned 10 times and the resultant radial separation was plotted (representative scan Fig. 1). Due to the fixed nature of the aggregation the resultant radial average and standard deviation are reported as bars in the main text. The FRET efficiency, E, was determined using by

$$E = 1 - \frac{F_d'}{F_d}$$

where F_d, is the emission intensity from the donor fluorophore, F_d', the emission after the acceptor is introduced. Due to the dipole coupling, the FRET efficiency was related to the average donor-acceptor separation, *r*, by [5, 6]

$$E = \frac{1}{1 + \left(r/R_0\right)^6}$$

were $\underline{r}$ is the average donor-acceptor separation, and R_o is the minimum separation required for fluorescence resonance energy transfer. To determine the degree of aggregation of protein within a solid scaffold, *E* was measured to determine the average relative distance, *r*, between two labeled proteins and the degree of aggregation determined as follows [5, 6].

$$r = R_0\left(\frac{1}{E} - 1\right)^{1/6}$$

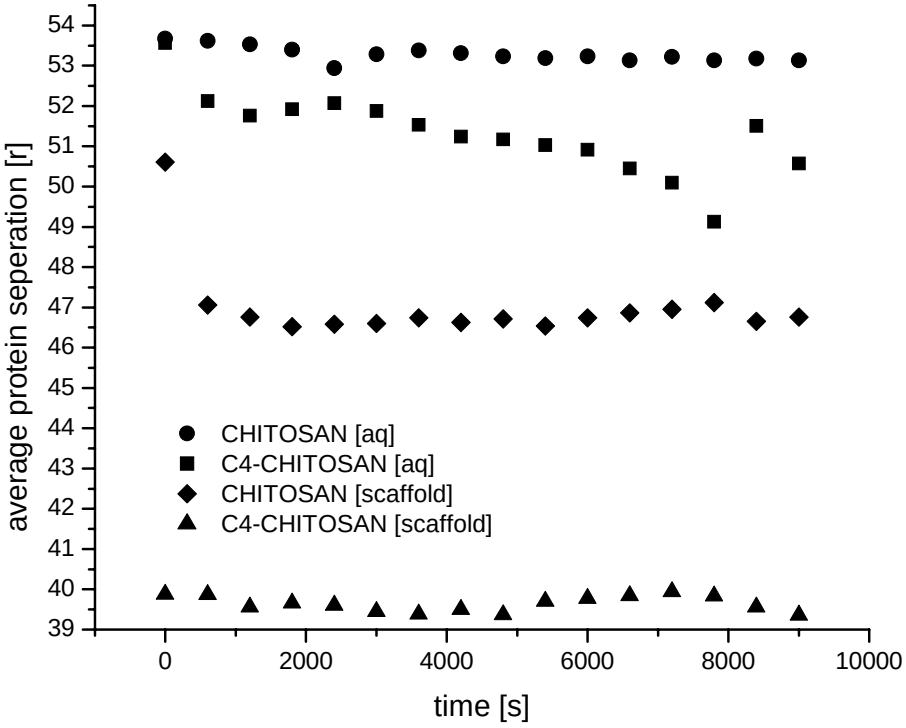

Figure 1 – Calculated radial separation as a function of time over the span of 3.5 hrs in 5min increments (not all data shown) for a solution of 50% *Alexa*®*555* tagged cMDH (30mM) and 50% *Alexa*®*647* tagged cMDH (30mM) in ● – aqueous chitosan, ■ – aqueous C4 – chitosan, ♦ – freeze dried chitosan scaffolds, and ▲ – freeze dried C4 – chitosan scaffolds.

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