Supporting Information

Mapping microclimate pH distribution inside protein-encapsulated PLGA microspheres using confocal laser scanning microscopy

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Analysis of Deviation in pH Distribution from Confocal Images

To assess errors that may be caused from generating the pixel-by-pixel pH map from confocal images, the following procedures were performed. First, the acquired confocal images (n=8) of standard pH buffer solutions were processed as reported previously[20]. The resulted image is a 512×512 matrix of pixel intensities. Then, by taking the ratio of pixel intensities of processed image at two emission wavelengths (I_{450nm}/I_{520nm}), a ratio matrix was generated. Next, each ratio pixel was converted to a pH according to the standard curve of intensity ratio *vs.* pH. After that, the probability density function of pH was fit with Gaussian function to obtain mean and standard deviation of pH distribution.

Table 1 displayed the variability of pH distribution yielded from this ratiometric measurement of confocal images. Except for the pH approaching detection limit (pH 5.8), in presence or absence of protein, the mean of measured pH was very closed to the actual pH. All pH distributions of standard solutions have a narrow Gaussian distribution with reasonably low standard deviation. Therefore, pH could be accurately mapped within the deviation range of ± 0.2 pH unit over pH from 2.8 to 5.8.

Table 1. pH distribution of mapped image from standard pH solution

BSA Concentration	**0 mg/ml**					**100 mg/ml**				
pH [a]	2.87	3.38	4.15	4.92	5.77	2.88	3.39	4.23	4.96	5.76
Mean pH [b]	2.92	3.36	4.13	4.91	5.60	2.91	3.31	4.20	4.90	5.61
SD [b]	0.10	0.04	0.04	0.07	0.17	0.04	0.03	0.01	0.07	0.15

[a] pH of the standard buffer solutions used to establish the standard curve of intensity ratio *vs.* pH
[b] mean pH and standard deviation were determined by fitting the pH distribution curve of standard pH solutions with Gaussian distribution function.
Dye concentration in the standard solutions was 1.2 mg/ml.

Interpolation of Standard Curves from Estimated Protein Concentrations

As shown in Figure S1, the fluorescence ratio increased with increasing the BSA concentration at constant pH. For BSA concentration from 0 to 250 mg/ml, a linear relationship of ratio *vs.* concentration could be assumed in the range of 0 to 25 mg/ml, 25 to 100 mg/ml, and 100 to 250 mg/ml. BSA concentration higher than 250 mg/ml is rare except after 1-day incubation, in that case, standard curves were fitted from experimental data.

Equations for standard curves of known concentration from experiment:

BSA=0 mg/ml, $y_0=-0.0572x^3+0.7352x^2-2.7529x+3.4356$

BSA=25 mg/ml, $y_1=-0.0731x^3+0.911x^2-3.2801x+3.91$

BSA=100 mg/ml, $y_2=-0.0769x^3+0.9682x^2-3.4631x+4.1084$

BSA=250 mg/ml, $y_3=-0.0935x^3+1.1604x^2-4.0344x+4.5951$

Where x is the pH and y is the fluorescence ratio.

If BSA concentration falls to the range of 25 to 100 mg/ml, the slope of linearity between concentration and fluorescence ratio (k) is given by:

$$k = \frac{y_2 - y_1}{100\,mg/ml - 25\,mg/ml}$$

$$= \frac{(-0.0769x^3 + 0.9682x^2 - 3.4631x + 4.1084) - (-0.0731x^3 + 0.911x^2 - 3.2801x + 3.91)}{75\,mg/ml}$$

$$= \frac{-0.0038x^3 + 0.0572x^2 - 0.183x + 0.1984}{75\,mg/ml}$$

For a calculated concentration α mg/ml, the corresponding standard curve can be predicted as:

$$y = y_2 - k \times (100\,mg/ml - \alpha\,mg/ml)$$

For example, if α=75 mg/ml, then

$$y = (-0.0769x^3 + 0.9682x^2 - 3.4631x + 4.1084) - \frac{-0.0038x^3 + 0.0572x^2 - 0.183x + 0.1984}{75\,mg/ml}$$

$$\times(100\,mg/ml - 75\,mg/ml)$$

$$= -0.0756x^3 + 0.9491x^2 - 3.4021x + 4.0423$$

The predicted curve was well aligned to the experimental curve. (Figure S2). Standard curves of concentrations fall within other ranges (0-25 mg/ml, 100-250 mg/ml) could be obtained similarly.

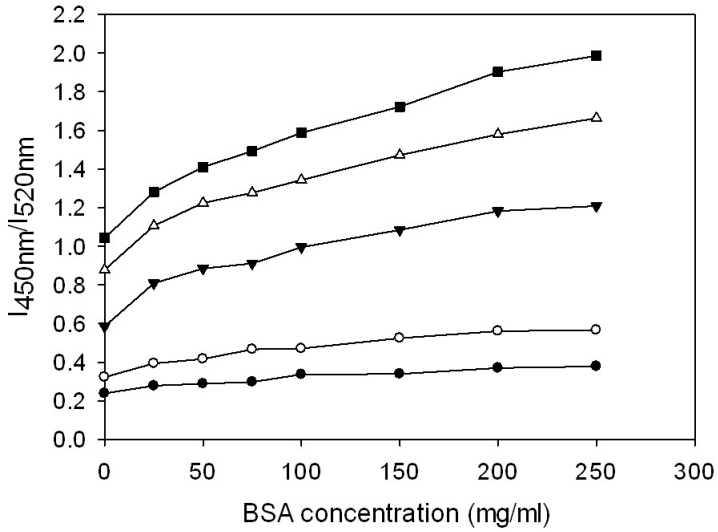

Figure S1. The BSA concentration dependency of fluorescence intensity ratio of Lysosensor yellow/blue® dextran at pH 2.8 (●), 3.4 (○), 4.2 (▼), 4.9 (△) and 5.7 (■). The concentration of dye was 1.2 mg/ml.

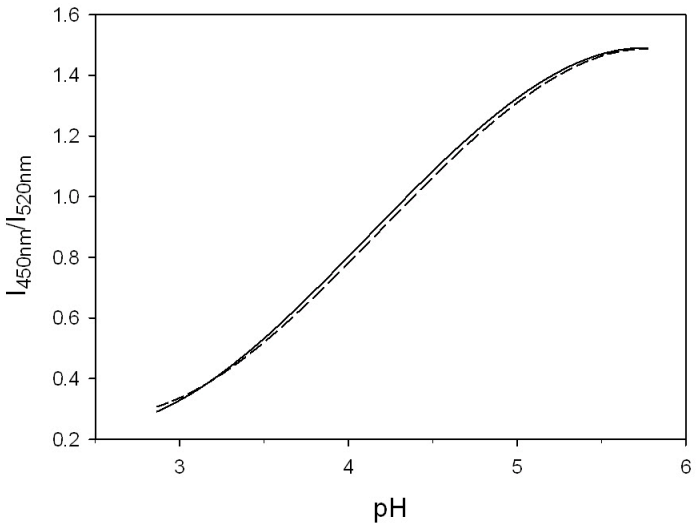

Figure S2. The pH sensitivity curves of Lysosensor yellow/blue® dextran in presence of 75mg/ml BSA plotted from fitting experiment data (— solid line) and predicted equation (-- dashed line).

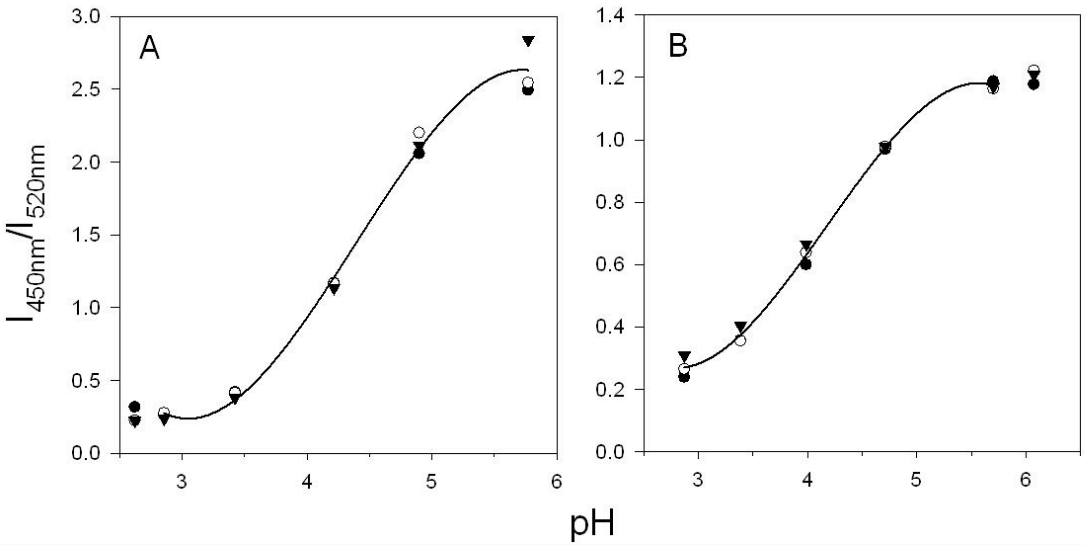

Figure S3. The pH sensitivity of confocal pH measurement of Lysosensor yellow/blue® dextran at concentration of 0.8 mg/ml (●), 1.2 mg/ml (○), and 2.0 mg/ml (▼) in presence of 100 mg/ml of BSA **(A)** and lysozyme **(B).** Lines represent best fits to a third order polynormial function of experimental data.

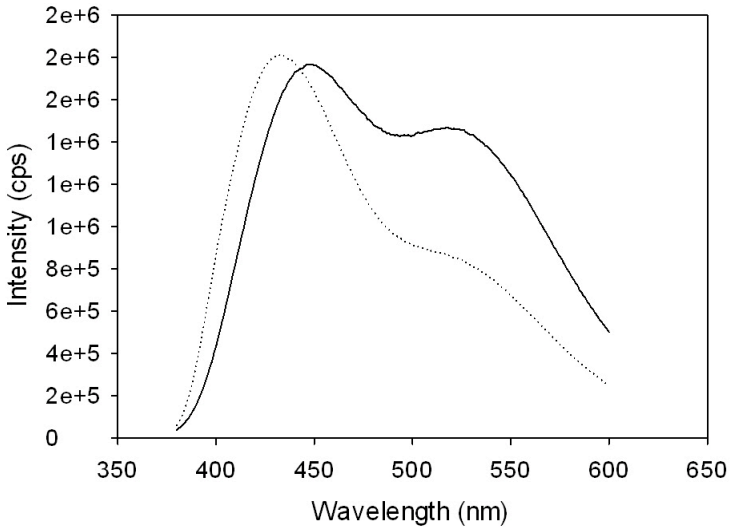

Figure S4. Fluorescence spectrum of Lysosensor yellow/blue® dextran in the absence (— solid line) and presence of 10 mg/ml BSA (--dashed line) in PBST (pH=7.4). The concentration of dye was 1.0 mg/ml.

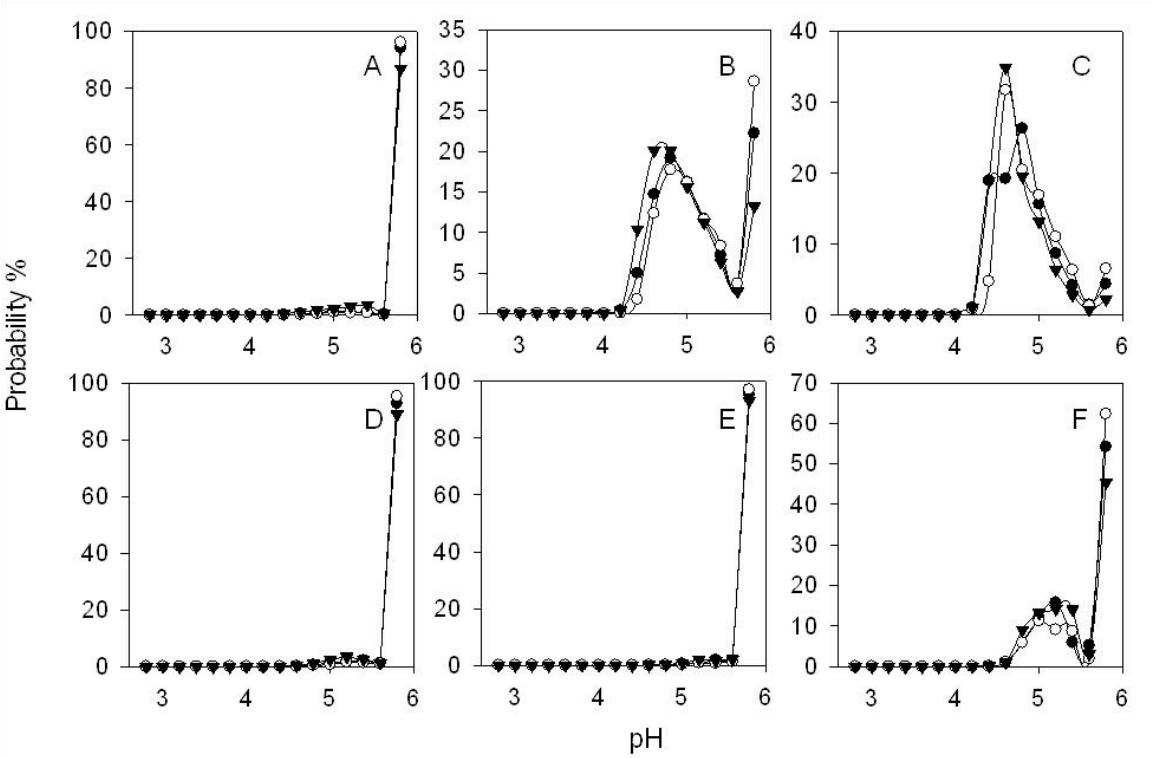

Figure S5. Comparison of μpH kinetics in microspheres estimated from protein concentration calculated from measured water uptake (●), 120% of measured water uptake (○), 80% of measured water uptake (▼) at 1 day **(A, D),** 14 days **(B, E)** and 28 days **(C, F)**. Microspheres were prepared from 40% (w/v) PLGA **(A-C)** and 40% (w/v) PLGA + $MgCO_3$ **(D-F)**.