## Supplementary Information for Dual Core Quantum Dots for Highly Quantitative Ratiometric Detection of Trypsin Activity in Cystic Fibrosis Patients

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# Analysis of trypsin activity using single colour quantum dot@silica-FRET nanosensors (1nanoSi): Proof-of-concept.

Figure S2 shows the photoluminescence (PL) absorption-emission profiles of the TAMRA dye and the  $QD_{540}$  quantum dots, respectively. The fluorescence emission from the  $QD_{540}$  overlaps very well with the absorption peak of TAMRA thus allowing the FRET process.<sup>1,2</sup>

Once the overlap between QD<sub>540</sub> and the fluorescent dye was confirmed, we carried out the encapsulation of quantum dots and the assembling between the QD@silica nanospheres and the TAMRA-labelled peptide. Thereafter, we performed the enzyme digestion using trypsin and read-out the emission of the 1nanoSi system. We used two different labelled peptides in order to obtain a control sample for our experiments. On one hand, the "*pro-active*" labelled peptide has the following sequence: 3'-NH<sub>2</sub>-Cys-Lys-Arg-Val-Lys-TAMRA-5'. Trypsin proteolytic activity is highly specific to the Lys-Arg-Val sequence and the enzyme digestions will cleave the peptide. On the other hand, our control sample, the "*in-active*" labelled peptide has the same chemical nature except for a change by Proline (Pro) instead of Arginine (Arg). Needless to say that trypsin cannot digest the small "*in-active*" peptide and the 1nanoSi fluorescence emission properties will remain the same. The 1nanoSi system was incubated with trypsin for the peptide digestion, and the recorded emission spectra are shown in Fig. S3. The spectroscopic profiles further demonstrated the existence of FRET processes between the  $QD_{540}$ @silica nanospheres and the "*pro-active*" TAMRA-labelled peptide (Fig. S3 a). Moreover, our control sample with the "*in-active*" 1nanoSi system, as expected, does not show any significant change in the fluorescence emission spectra (Fig. S3 b).

Fig. S4 displays the emission spectra of the 2nanoSi nanospheres functionalized with different concentrations of the "*pro-active*" TAMRA-labelled peptide (400-4000 µg/L) in order to see how the amount of dye affects the emission of QDs. First, we would like to highlight the blue shift in the PL of QDs@silica in comparison to the pristine QDs in solution, i.e. 510 nm versus 540 nm, as observed previously,<sup>3,4,5</sup> yet the original fluorescence is recovered after the trypsin digestion as shown later in this work. Increasing the amount of TAMRA-labelled peptides on the QD<sub>540</sub>@silica nanospheres surface leads to a decrease in the emission intensity between  $\lambda = 450$  nm and 550 nm range, which corresponds to the fluorescence emission wavelength of the quantum dots, as mentioned above. The emission intensity drop is a direct evidence of efficient FRET between the QD<sub>540</sub>@silica nanospheres and the TAMRA dye in the 2nanoSi system, as reported previously in analogous FRET systems.<sup>6</sup> However, at higher concentration of TAMRA-labelled peptide, that is above 1800 µg/L, the FRET process is saturated.

#### FRET measurements and calculations.

The FRET efficiency (E) is the quantum yield of the energy transfer transition, *i.e.* the fraction of energy transfer event occurring per donor excitation event. The experimental efficiency, E is defined by the following equation:

 $E = (F_D - F_{DA})/F_D$ 

where  $F_D$  and  $F_{DA}$  are the fluorescence intensities of the donor alone and the donor in the presence of acceptor,<sup>7,8</sup> respectively.

The FRET efficiency relates to the quantum yield and the fluorescence lifetime of the donor molecule as follows:<sup>9</sup>

 $E=1-\tau'_D/\tau_D$ 

where  $\tau'_D$  and  $\tau_D$  are the donor fluorescence lifetimes in the presence and absence of an acceptor, respectively.

Based on the fluorescence measurements in Figure S4, we obtained the following efficiencies for the 400-4000  $\mu$ g/L concentrations range of the "*pro-active*" TAMRA-labelled peptide:

[peptide- TAMRA]	400	800	1200	1600	1800	2000	3000	4000
E (%)	20.45	47	59.61	68	72.83	72.96	72.90	72.96
n	0.3	1	1.67	2.36	3.03	3.05	3.04	3.05

Estimates of the QD donor-dye acceptor separation distance r were calculated via the Förster formalism by fitting the above experimental FRET efficiencies E using the expression:

 $E=1/[1+(r/R_0)^6]$ 

Where  $R_0$  is the Förster distance designating the donor-acceptor separation at 50% energy transfer efficiency.  $R_0$  (in Angstrom, Å) is expressed as:<sup>10</sup>

 $R_0 = 0.211 (\kappa^2 n_D^{-4} Q_D J (\lambda))^{1/6}$ 

where  $n_D$  is the refractive index of the medium,  $Q_D$  is the donor quantum yield in the absence of acceptor,  $J(\lambda)$  is the spectral overlap integral, and  $\kappa^2$  is the dipole orientation factor. We use  $\kappa^2 = 2/3$  corresponding to a random dipole orientation shown to be appropriate for our self-assembled QD–protein/peptide–dye conjugates, as detailed in reported previous studies.<sup>6,8</sup> This is based on the assumption that in a self assembled QD–peptide/protein–dye pair it is impossible to control the relative orientation of the dipoles. Each time a dye-labelled protein it is added to the conjugate, that dye will have a dipole orientation that does not correlate with the existing QD and dyes.

For our system, we obtained a  $R_0 = 4.982$  nm and r = 5.10 nm at 47% energy transfer efficiency by fluorescence.

#### Dependence of *E* on the number of acceptors, *n*.

Supposing spherical symmetry of the dye-labelled peptides attached to the QDs@silica nanosphere surface, the number of acceptors n can be calculated following the Förster model:<sup>10</sup>

#### $E = n/[n+(r/R_0)^6]$

Considering the Förster distance  $R_0 = 4.982$  nm and r = 5.10 nm, then the FRET efficiency of approx. 50% will correspond to a single donor-acceptor pair, while the FRET saturation (approx. 73%) is reached at three dye-labelled peptides on the surface of the QDs@silica nanosphere (Figure S5).

As a consequence, the concentration of 800  $\mu$ g/L of TAMRA-labelled peptide was chosen for all the enzymatic experiments as described in the main manuscript and the corresponding supporting information.

#### Michaelis-Menten kinetics.

Values for  $K_m$  and  $k_{cat}$  were determined from a conventional excess-substrate Michaelis-Menten (MM) plot as shown in Figure S6 and eq 1.

$$V = d[S]/dt = V[S]/(Km + [S]) = k_{cat} [E]_0 [S] / (k_1^{-1}(k_{-1} + k_{cat}) + [S])$$
(1)

The terms inlcude [S], the concentration of substrate; V, the máximum rate of catalysis;  $K_{\rm m}$ , the Michaelis constant;  $k_{\rm cat}$ , the turnover number;  $k_1$  and  $k_{-1}$ , the rates for ES complex association and dissociation; and [E]<sub>0</sub>, the total concentration of enzyme. <sup>11,12</sup>

Each QDs@silica-TAMRA-labelled peptide substrate conjugate was digested with 150  $\mu$ g/L of trypsin and the data was collected for 10 min. The MM kinetic parameters were determined as following:

$$K_{\rm m} = 3.5 \text{ mM}$$
  
 $k_{\rm cat} = 50.4 \text{ s}^{-1}$   
 $k_{\rm cat}/K_{\rm m} = 14.39 \text{ mM}^{-1} \text{ s}^{-1}$ 

Taking into account the kinetic parameters for the natural substrate trypsinogen ( $K_{\rm m} = 5.1 - 5.6 \,\mu\text{M}$ ,  $k_{\rm cat} = 2.12 - 4 \,\text{s}^{-1}$  and  $k_{\rm cat}/K_{\rm m} = 41 \,\mu\text{M} \,\text{s}^{-1}$ ),<sup>13,14</sup> our results are different. However, considering other kind of substrates, such as peptides, our results fit rather well with these studies relating the affinity of trypsin with the different peptidic sequences ( $K_{\rm m} = 1.9 - 4.7 \,\text{mM}$ ,  $k_{\rm cat} = 7.1 - 290 \,\text{s}^{-1}$ ).<sup>15</sup>

#### Stool trypsin concentration in the biological samples.

The 2nanoSi system was used for quantifying trypsin concentration in faecal samples from 4 CF patients (subjects C, F, I and K in Table S1), and 7 healthy subjects, which are heterozygotic for non-functional CFTR gene (subjects A, B, D, E, G, H and J in Table S1). Trypsin concentration in these samples was determined based on the calibration curve plotted in the coordinates  $I_{540}/I_{660}$  versus trypsin concentration (Fig. 3). The results are shown in Table S1 and confirm that the faeces of the 4 CF patients contained stool trypsin concentration below 90 µg/g faeces, while non-CF patients had faecal trypsin concentration above 90 µg/g.

Table S1. Mean values (as-measured and corrected\*) and standard deviation for the biological samples. Each stool sample was measured 5 times. Corrections were applied in the case of the subjects with supplementary enzymatic medication.

Subject —	Trypsin (μg/g)						
Subject	as-me	asured	corrected*				
Α	171,98	± 3,081	171,98	± 3,081			
В	121,92	± 1,504	121,92	± 1,504			
С	129,19	± 4,053	40,05	± 1,256			
D	217,01	± 8,341	217,01	± 8,341			
Е	188,04	± 7,277	188,04	± 7,277			
F	121,15	± 4,694	10,9	± 0,422			
G	168,58	± 9,997	168,58	± 9,997			
Н	158,85	± 7,767	158,85	± 7,767			
Ι	111,89	± 8,272	34,69	± 2,564			
J	133,41	± 3,043	133,41	± 3,043			
K	128,93	± 4,905	50,28	± 1,913			

It has to be taken into account that these four particular CF patients (subjects C, F, I and K, respectively) were taking supplementary pancreatic enzymes (8-10 capsules of Creon© 25,000 or 20 capsules of Creon© 10,000 daily, respectively), and consequently, the additional enzymatic activity provided by such medication had to be subtracted from the as-measured trypsin concentration (see Table SI 2).

Subject	С	F	Ι	K			
Medication	Creon 25,000 <sup>a</sup>	Creon 10,000 <sup>b</sup>	Creon 25,000	Creon 25,000			
Trypsin / capsule <sup>b</sup>	1,000	600	1,000	1,000			
Capsules / day	9	20	9	8			
Total trypsin /	0.000	12 000	0.000	8 000			
day <sup>c</sup>	9,000	12,000	9,000	8,000			
Minimal							
functional trypsin	13,000						
in the body <sup>d</sup>							
Extra trypsin	700/	020/	700/	620/			
from medication <sup>e</sup>	/0/0	9270	/0/0	0270			
Real trypsin in	200/	Q0/_	200/	280/			
the body <sup>f</sup>	3070	0 / 0	5070	3870			
[trypsin] as-	120.10	121 15	111.80	128.02			
measured $(\mu g/g)^g$	129,19	121,13	111,09	126,95			
[trypsin]	10.05	10.0	24.60	50.28			
corrected $(\mu g/g)^h$	40,03	10,9	54,09	50,20			

Table S2. Calculations for corrected faecal trypsin concentration in CF patients.

<sup>a</sup>Creon<sup>®</sup> 25000 Capsules. Each capsule contains: lipase 25,000 PhEur units, amylase 18,000 PhEur units, trypsin 1,000 PhEur units.

<sup>b</sup>Creon<sup>®</sup> 10000 Capsules. Each capsule contains: lipase 10,000 PhEur units, amylase 8,000 PhEur units, trypsin 600 PhEur units.

<sup>c</sup>Total trypsin / day = number of capsules per day \* trypsin per capsule

<sup>d</sup>Normal trypsin value in healty subjects

<sup>e</sup>Extra trypsin from medication = [total trypsin per day / minimal functional trypsin in the body] \* 100

<sup>f</sup>Real trypsin in the body = 100% - extra trypsin from medication

<sup>g</sup>Trypsin determined using our model

<sup>h</sup>Trypsin value corrected = real trypsin in the body \* trypsin determined using our model

### Supplementary figures and tables



Fig. S1 Emission spectra of control samples excited at 405 nm: CdSe<sub>540</sub>@silica (green), CdSe<sub>660</sub>@silica (red) and 5'-TAMRA (orange), respectively. All the samples were measured at the same window aperture conditions.



Spectral overlap integrand (J) (M^-1 cm^-1 nm^4)



Fig. S2 Top, absorption of 5'-TAMRA (red) and emission spectra of  $QD_{540}$  quantum dots (blue), and their corresponding spectral overlap (bottom).



Fig. S3 Emission spectra of 5'TAMRA-labelled peptide-functionalized  $QD_{540}$ @silica nanospheres after trypsin digestion (a) the "*pro-active*" 1nanoSi and (b) the "*in-active*" 1nanoSi. The 1nanoSi system was incubated with trypsin (250 µg/mL) and the figure legends correspond to the enzymatic digestion time in minutes (0-30 min).



Fig. S4 Emission spectra of "*pro-active*" 2nanoSi system increasing the amount of TAMRA-labelled peptide at the surface of the nanosphere in the range 400-4000  $\mu$ g/L.



Fig. S5 Dependence of the FRET efficiency on the number of acceptors.



Fig. S6 Michaelis-Menten (MM) plot for TAMRA-labelled peptides digestion (•), catalyzed by 150 ug/L of trypsin. The blue line is the fit to the integrated MM model.



Fig. S7 Emission spectra of  $QD_{540}$  (green), 5'-TAMRA (orange), and  $QD_{660}$  (red). The quantum dots were excited at 405 nm, while the TAMRA dye was excited at 535 nm.





Fig. S8 Emission spectra of 5'TAMRA-labelled 2nanoSi nanospheres. Enzymatic digestion with different trypsin concentration: a) 250  $\mu$ g/L, b) 200  $\mu$ g/L, c) 100  $\mu$ g/L, and d) 50  $\mu$ g/L, respectively.



Fig. S9 Biological samples measured 5 times from stool concentration of trypsin for each subject with the range of the measured values and standard deviations in a) asmeasured and b) corrected values for the supplementary enzymatic activity subtraction.

#### Notes and references

1 R. Clegg, R. in *FRET and FLIM techniques*. *Laboratories techniques in biochemistry and molecular biology*, Vol. 33 (Ed.: T. W. J. Gadella) Elsevier, Amsterdam, 2009.

2 R. C. Somers, R. M. Lanning, P. T. Snee, A. B. Greytak, R. K. Jain, M. G. Bawendi and D. G. Nocera, *Chem. Sci.*, 2012, 3, 2980.

3 S. P. Wang, N. Mamedova, N. A. Kotov, W. Chen and J. Studer, *Nano Lett.*, 2002, **2**, 817.

4 I. Castelló Serrano, Q. Ma and E. Palomares, E. J. Mater. Chem., 2011, 21, 17673.

5 Q. Ma, I. Castelló Serrano and E. Palomares, Chem. Commun., 2011, 47, 7071.

6 A. M. Dennis, W. J. Rhee, S. N. Dublin, D. Sotto, G. Bao, ACS Nano, 2012, 6, 2917.

7 I. L. Medintz, S. A. Trammell, H. Mattoussi and J. M. Mauro, J. Am. Chem. Soc. 2004, **126**, 30.

8 I. L. Medintz, J. H. Konnert, A. R. Clapp, I. Stanish, M. E. Twigg, H. Mattoussi, J. M. Mauro and J. R. Deschamps, *PNAS*, 2004, **101**, 9612.

9 I. Majoul, Y. Jia, R. Duden, in *Handbook of Biological Confocal Microscopy*, (Ed.: J. B. Pawley) Springer, New York, 2006.

10 J. R. Lakowicz in *Principles of Fluorescence Spectroscopy*, (Ed.: J. R. Lakowicz) Kluwer Academic/ Plenum, New York, 1999.

11 D. C. Purich in *Enzyme Kinetics: Catalysis & Control*, (Ed.: D. C. Purich) Elsevier, Burlington, 2010.

12 G. E. Briggs and J. B. S. Haldane, Biochem. J., 1925, 19, 338.

13 F. J. Castillo-Yáñez, R. Pacheco-Aguilar, F. L. García-Carreño and M. A. Navarrete-Del Toro, *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 2005, **140**, 91.

14 D. Lu, X. Yuang, X. Zheng and J. E. Sadler, J. Biol. Chem., 1997, 272, 31293.

15 R. M. Caprioli and L. Smith, Anal. Chem., 1986, 58, 1080.