Electronic Supplementary Information

A FRET-based biosensor for the detection of neutrophil elastase

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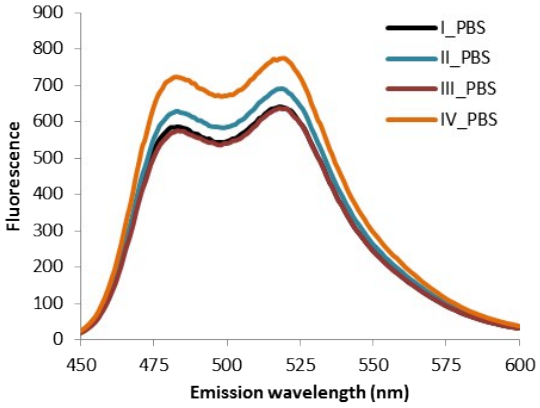

Fig. 1†. Fluorescence emission spectra of the four biosensors in PBS upon excitation at 428 nm.

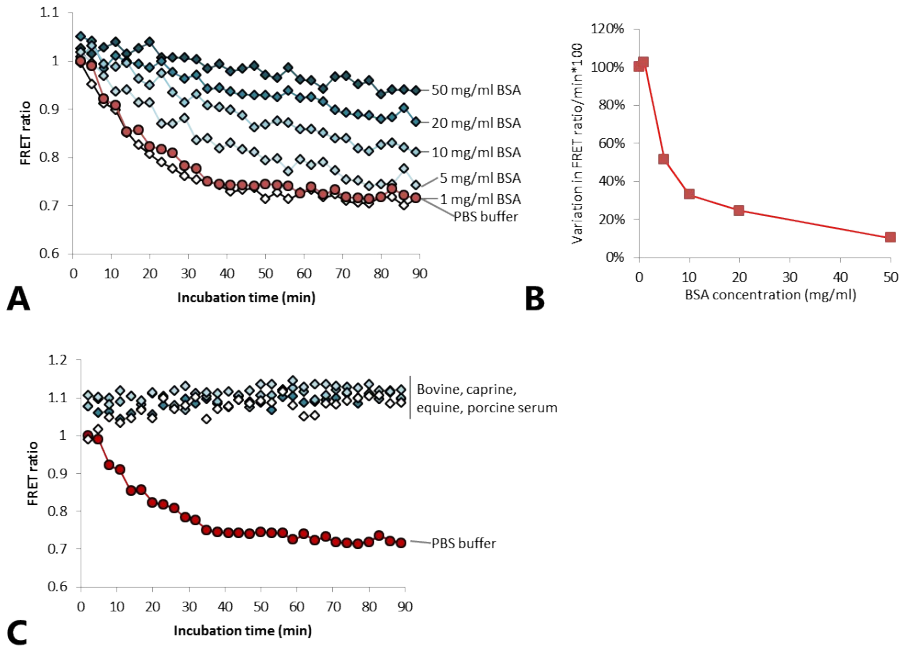

Fig. 2†. Influence of BSA on neutrophil elastase activity. The protein biosensor was incubated with NE in the presence of different amounts of BSA in PBS buffer and the FRET ratio was monitored over time (A). The variation in FRET ratio over time upon addition of NE was calculated from the initial linear part of the curve by linear regression (n=1) (B). Similarly, the effect of a complex protein mixture was tested by incubating the protease biosensor with NE and 10% v/v serum from cow, goat, horse and pig (n=1) (C).

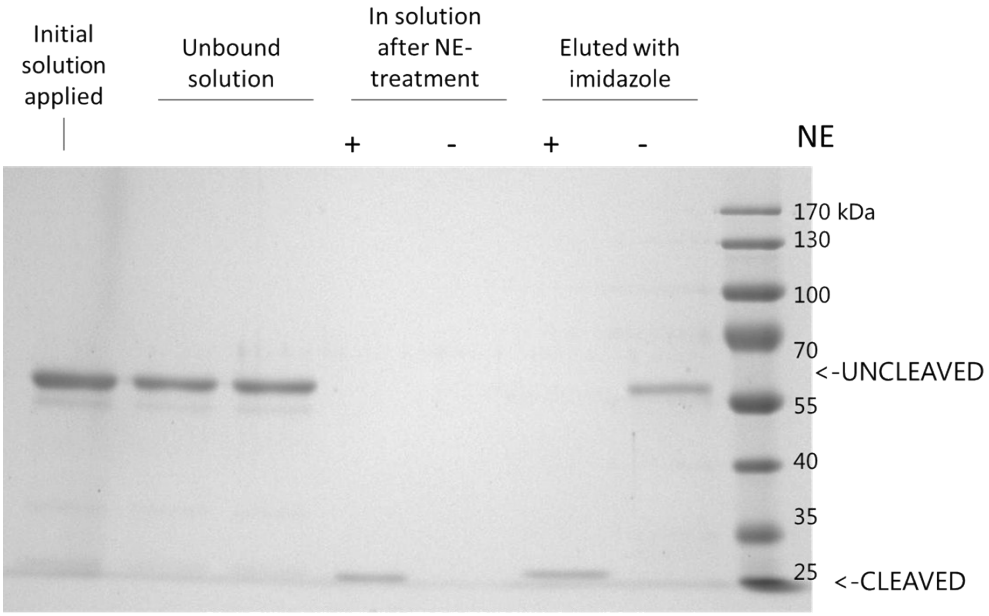

Fig. 3†. Immobilization of biosensor 4 to nickel-NTA coated plate. SDS PAGE analysis of the applied solution containing biosensor IV, the not-immobilized fraction, the portion released upon NE-treatment, and the immobilized portion after elution with imidazole.

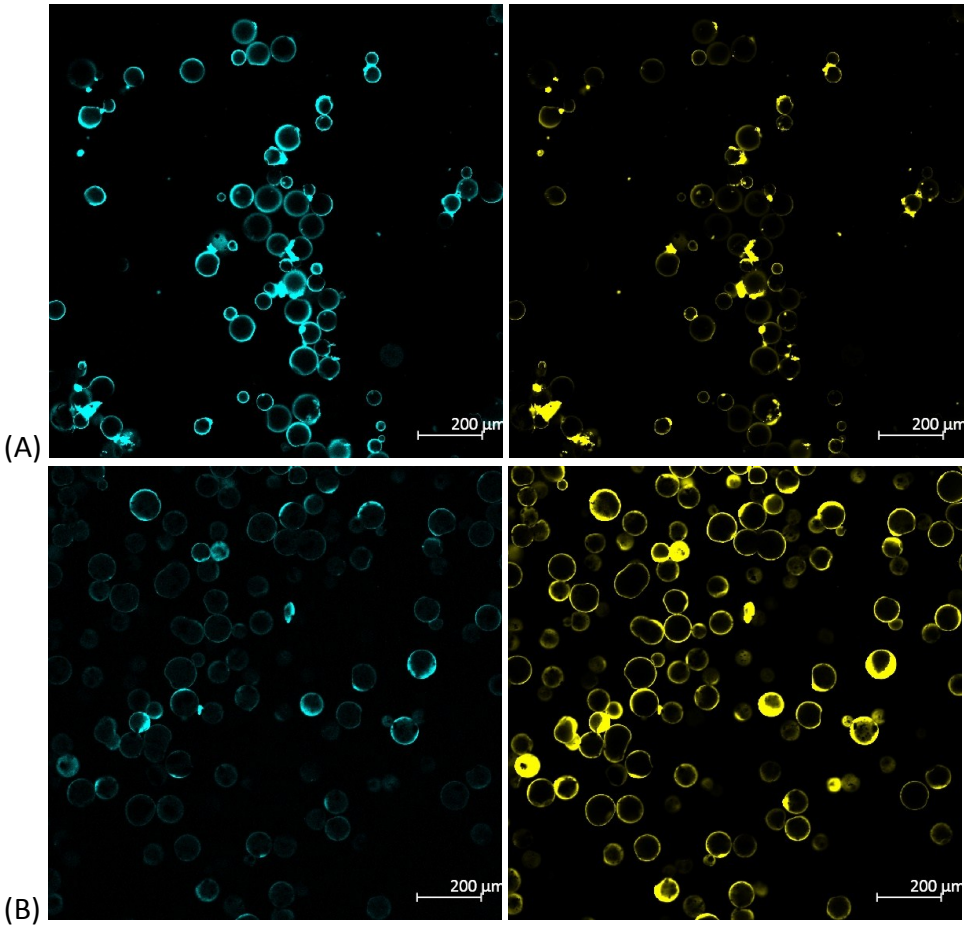

Fig. 4†. Confocal microscopy of superparamagnetic microparticles carrying the biosensor before (A) and after (B) incubation with NE. In blue: $\lambda_{ex.}$= 458 nm, $\lambda_{em.}$= >486 (donor only) In yellow: $\lambda_{ex.}$ =514 nm, $\lambda_{em.}$ =>570 (acceptor only).

Table 1†. Influence of the linker length on the FRET ratio of the biosensors before and after treatment with neutrophil elastase in PBS buffer, DMEM, and artificial wound exudate (AWE).

		FRET ratio			
	NE treatment	Sensor I	Sensor II	Sensor III	Sensor IV
PBS buffer	no	1.00±0.01	1.01±0.01	1.02±0.01	1.02±0.01
	yes	0.69±0.01	0.69±0.01	0.69±0.01	0.69±0.01
DMEM	no	0.97±0.01	0.98±0.01	0.96±0.02	0.98±0.01
	yes	0.69±0.01	0.69±0.01	0.69±0.01	0.69±0.01
AWE	no	1.07±0.01	0.97±0.08	1.08±0.01	1.11±0.01
	yes	0.99±0.08	1.07±0.01	1.08±0.01	1.09±0.02

Table 2†. Primer used for CFP-NE-YFP construction.

Primer name	Primer sequence
CFP_Y66Wfwd	CACTACTTTCTCTTGGGGTGTTCAATGCTTTTCC
CFP_Y66Wrev	AAGCATTGAACACCCCAAGAGAAAGTAGTGACAAG
YFP_T203fwd	ATTACCTGTCGTATCAATCTGCCCTTTCGAAAGATCCC
YFP_T203rev	AAGGGCAGATTGATACGACAGGTAATGGTTGTCTGG
GFP3link	GGCATCCGGTTACGGTATGCACTAGTTTTGTAGAGCTCATCCATGCC
GFP5link	GCATACCGTAACCGGATGCCACCGGTAGTAAAGGAGAAGAACTTTTCAC
NE_01fwd	CTAGTGGTGGTTCTCGTCAGTTCATCCGTTGGGGTGGTGGTGGTTCTA
NE_01rev	CCGGTAGAACCACCACCACCCCAACGGATGAACTGACGAGAACCACCA
NE_02fwd	CTAGTCGTCAGTTCATCCGTTGGGGTGGTA
NE_02rev	5`CCGGTACCACCCCAACGGATGAACTGACGA
NE_03fwd	CTAGTTTCATCCGTTGGA
NE_03rev	CCGGTCCAACGGATGAAA
NE_04fwd	CTAGTGGTGGTTCTGGTGGTACCCGTCAGTTCATCCGTTGGGGTGGTGGTGGTTCTGGTGGTGGTA
NE_04rev	CCGGTACCACCACCAGAACCACCACCACCCCAACGGATGAACTGACGGGTACCACCAGAACCACCA

Experimental Section

Construction of the four biosensor variants: Molecular cloning was performed according to standard procedures. Oligonucleotide synthesis and DNA sequencing were carried out by Microsynth (Balgach, Switzerland). The genes coding for unconjugated CFP and YFP were assembled with the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies) using the gene coding for GFP as a template and the primer CFP_Y66Wfwd and CFP_Y66Wrev for CFP construction and the primer YFP_T203fwd and YFP_T203rev for YFP construction (Table S1). The gene coding for CFP was subsequently amplified by using the T7 forward primer (Microsynth, Balgach, Switzerland) and the primer GFP3link (Table S1), removing the C-terminal His-tag and introducing a SpeI cleavage site at the 3´end of the CFP gene. The gene coding for YFP was amplified by using the T7 reverse primer (Microsynth, Balgach, Switzerland) and the primer GFP5link (Table S1), to introduce an AgeI cleavage site at the 5´end of the YFP gene. The two amplicons were subsequently connected *via* an overlap extension reaction. The combined fragment was digested using the NdeI and EcoRI restriction sited and cloned into the NdeI/EcoRI-cleaved pET22b(+) vector. Synthetic DNA fragments coding for four linker regions (I: NE_01fwd and NE_01rev; II: NE_02fwd and NE_02rev; III: NE_03fwd and NE_03rev; IV: NE_04fwd and NE_04rev (Table 2†) were purchased (Microsynth, Balgach, Switzerland) and directly ligated into the corresponding SpeI/AgeI-cleaved vectors. Strains *E. coli* XL1-Blue and *E. coli* strain BL21(DE3)gold (Stratagene) was used for cloning and for protein expression, respectively. For purification and immobilization purposes, a six-histidine-tag was engineered at the C-terminus of the biosensor molecule.

Protein production and purification: Chemocompetent BL21(DE3)gold cells were transformed with pET22b(+), with the inserted gene for the CFP-NE-YFP fusion construct.

500 ml LB medium containing 50 µg ml^{-1} ampicillin were inoculated with an overnight pre-culture to a starting OD_{600} of 0.05 and incubated at 37°C until an OD_{600} of 0.6-0.8 was reached. Gene expression was induced with 1 mM IPTG. Cells were harvested by centrifugation after 16 h growth at 18°C. The biomass was stored at -20°C. Cells were resuspended in 100 mM potassium phosphate buffer pH 7.0, including 10 mM imidazole, and incubated for 30 min at 4°C in presence of 1 µg ml^{-1} lysozyme and protease inhibitor cocktail (Roche, cOmplete, EDTA-free). After cell lysis by sonication, the solution was treated with 0.2 µl ml^{-1} Benzonase (NEB) for 30 min and centrifuged at 14000 rpm for 50 min at 4°C. The biosensors were purified *via* the C-terminal hexa-histidine tag by fast protein liquid chromatography (Äkta Purifer, GE Healthcare) with a HisTrap HP column (GE Healthcare) pre-equilibrated with 100 mM potassium phosphate buffer pH 7.0, 10 mM imidazole. The protein was eluted with a gradient of 10 to 500 mM imidazole. Biosensor-containing fractions were concentrated with a 3 kDa Amicon Ultra-15 Centrifugal Filter Unit (Millipore) and injected onto a Superdex 75 10/300 GL (GE Healthcare) size exclusion column pre-equilibrated with PBS buffer pH 6.2. Protein purity was determined by SDS PAGE.

Determination of protein concentration and sequence analysis: Protein concentrations were determined by absorbance spectroscopy using the extinction coefficient at 280 nm calculated by the Protparam software at ExPASY (http://web.expasy.org/protparam/), e.g. 55030 $M^{-1}cm^{-1}$ for the variant IV. N-terminal sequencing was carried out by Edman degration by the Functional Genomics Center Zurich (FGCZ, Zürich, Switzerland). Putative protease sites were identified using the PROSPER software (https://prosper.erc.monash.edu.au)

Calculation of the FRET ratio: FRET ratio was determined by fluorescence spectroscopy using 0.2 mg ml^{-1} of each construct. Ratios were measured in PBS buffer pH 6.2, Dulbecco's modified eagles medium (DMEM) (product nr. D5030, Sigma-Aldrich) solubilized in PBS

buffer pH 6.2, or artificial wound exudate (AWE) that was prepared by adding 10% porcine serum (Gibco, Life Technologies) to DMEM solubilized in PBS buffer pH 6.2. The donor was excited at 428 nm with a band width of 5 nm. The emission was measured from 450 to 600 nm with a band width of 10 nm. The FRET ratio was determined as emission intensity ratio of the emission of YFP at 528 nm and CFP at 458 nm.

Degradation kinetics with neutrophil elastase: Kinetic measurements were performed at room temperature using substrate concentrations of 0.01 to 0.8 mg ml^{-1} and enzyme concentrations of 250 to 50 ng ml^{-1} in PBS buffer pH 6.2. For this NE (Elastase from human leucocytes, product nr. E8140-1UN, Sigma Aldrich) was dissolved in 200 µl PBS buffer. For measurements 10 µl of the 50-fold diluted enzyme solution (0.1 U $µl^{-1}$) was used on 150 µl reaction mixtures. Degradation was followed by fluorescence spectroscopy. The donor was excited at 428 nm with a band width of 5 nm. The emission was measured from 450 to 600 nm with a band width of 10 nm. The FRET ratio was determined as emission intensity ratio of the emission of YFP at 528 nm and CFP at 458 nm. The data were fitted by a single exponential equation. To determine the steady-state parameter k_{obs} and K_M, varying substrate concentrations were incubated with ~20 ng ml^{-1} NE. The initial velocities were plotted as a function of substrate concentration and the data were fitted using the Michaelis-Menten equation. To analyze the cleaved sample, the protein (0.2 mg ml^{-1}) was incubated for 90 min in presence of ~50 ng ml^{-1} NE before SDS-PAGE analysis.

Immobilization of the biosensors: The biosensors were immobilized on a 96-well polystyrene HIS-Select® High Capacity (HC) Nickel coated plate (Product nr. S5563, Sigma Aldrich, Buchs, Switzerland). 200 µl of each protein was incubated in a well at a 0.1 mg ml^{-1} concentration for 1 hour at 25°C, in PBS pH 6.2. After removing the unbound solution, each well was rinsed 3 times with 200 µl PBS pH 6.2. Digestion with NE was carried out by adding 5 µl of the NE

stock (0.5 mU) and 195 µl of buffer and incubating for 1 h at 25°C under gentle shaking. The solution was then removed and the immobilized protein eluted with PBS pH 6.2 supplemented with 100 mM imidazole. For immobilization to superparamagnetic particles, 8 µl of HIS-Select® Nickel Magnetic Agarose Beads (sigma Aldrich, Buchs, Switzerland) were first rinsed twice by resuspension in 200 µl 20 mM MOPS pH 6.2 and collection with a magnet, and then incubated in the presence of 0.4 mg/ml biosensor variant I for 10 min, 800 rpm, 22°C. Particles were collected with a magnet to remove the unbound protein fraction, resuspended in 100 µl 20 mM MOPS pH 6.2 and later added of 10 µl NE solution or buffer (negative control).

Microscopy: Confocal laser scanning microscopy (CLSM) of microparticles resuspended in 20 mM MOPS pH 6.2 was carried out using a LSM 780 from Carl Zeiss Microscopy (Jena, Germany).