Supporting Information

Label-free microfluidic free-flow isoelectric focusing, pH gradient sensing and near real-time isoelectric point determination of biomolecules and blood plasma fractions

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S1 Materials and methods

S1.1. Chemicals

4-Acryloylmorpholine, olyethylenglycol-diacrylate (OEG-DA, MW 258 and 700), 2-hydroxy-4´-(2-hydroxy)-2-methylpropiophenone, 3-(trimethoxysilyl)propyl methacrylate (TPM), 2.2-dimethoxy-2-phenylacetophenone (DMPA, 99 %), phosphate buffered saline (PBS, 150 mM), trichloromethane, sorbitan monolaurate (Tween 20), ampicilin sodium salt, plasma from bovine, α-lactalbumin from bovine milk and β-lactoglobulin B from bovine milk were purchased from Sigma-Aldrich (Steinheim, Germany). H_3BO_3, H_3PO_3, CH_3COOH, NaOH, HCl, H_2SO_4, $NaHCO_3$ and *iso*-propanol were acquired from Merck (Darmstadt, Germany). Methanol (ROTISOLV ≥99.98 %, Ultra LC-MS) and formic acid (98 % p.a.) were purchased from Carl Roth (Karlsruhe, Germany). Ofloxacin was aquired from Dr. Winzer Pharma GmbH (Berlin, Germany). Carbonate buffer was made of 100 mM $NaHCO_3$ and the pH was adjusted to 9 by titration with 0.1M HCl.

Britton-Robinson buffer (BRB) was made of 10 mM H_3BO_3, H_3PO_3 and CH_3COOH and the pH was adjusted by titration with 1 M NaOH solution and monitored using a pH meter Lab 850 (SI Analytics, Mainz, Germany). Ampholyte pH 4-7 was acquired from AppliChem (Darmstadt, Germany). N-(3-([N-(3-(methacryloylamino)propyl) amino]sulfonyl)-2,6-diisopropylphenyl)-N'-(4-([N-(3-(methacryloylamino)propyl)amino] sulfonyl)-2,6-diisopropylphenyl)-1-(4-methyl-1-piperazinyl)-6,7,12-trichloroperylene-3,4,9,10-tetracarboxylic bisimide (Perylenebisimide, PBI) was synthesized by Daniel Aigner according to Ref. S1.

S1.2. Microfluidic free-flow isoelectric focusing (μFFIEF)

neMESYS syringe pumps (cetoni, Korbussen, Germany) were used for flow control in the separation bed. The electric field was induced with a HCP 35-6500 power supply (FuG Elektronik, Rosenheim, Germany) in negative mode. For incoupling of the electric field inside the microfluidic chip, platinum electrodes were inserted in the electrode inlets (Figure 1g) and connected with the power supply. For the FFIEF the analyte solution was injected through the center inlet of the microchip (flow rate: 0.5 $\mu L \cdot min^{-1}$ for the proteins, 1.0 $\mu L \cdot min^{-1}$ for the blood plasma and 1.5 $\mu L \cdot min^{-1}$ for the antibiotics). Next to the analyte the ampholyte flows (0.1 % ampholyte 4-7, 50 ppm (w/w) Tween 20, flow rate: 1.0 $\mu L \cdot min^{-1}$), the sheath flows (10 mM BRB pH 10 and 20 mM H_2SO_4 pH 1.4 respectively, flow rate: 3.0 $\mu L \cdot min^{-1}$ for the proteins and the plasma and 2.0 $\mu L \cdot min^{-1}$ for the antibiotics) and the electrode flows (flow rate: 5.0 $\mu L \cdot min^{-1}$ for the proteins, 2.0 $\mu L \cdot min^{-1}$ for the blood plasma and 3.0 $\mu L \cdot min^{-1}$ for the antibiotics) were applied. The cathodic flow consisted of 10 mM BRB pH 10 (8.05 $mS \cdot cm^{-1}$) and the anodic flow of 20 mM H_2SO_4 (pH 1.4).

S2. Spectroscopic measurements and sensor characterization

S2.1. Spectroscopic measurements of the sensor matrix and the biomolecules

Absorbance and emission spectra were recorded with a UV-Vis spectrometer (P650 from Jasco, Gross-Umstadt, Germany) and a spectrofluorometer (FP-6200 from Jasco, Gross-Umstadt, Germany). The measured spectra with the employed filters and light sources in the IEF are shown in Figure S1.

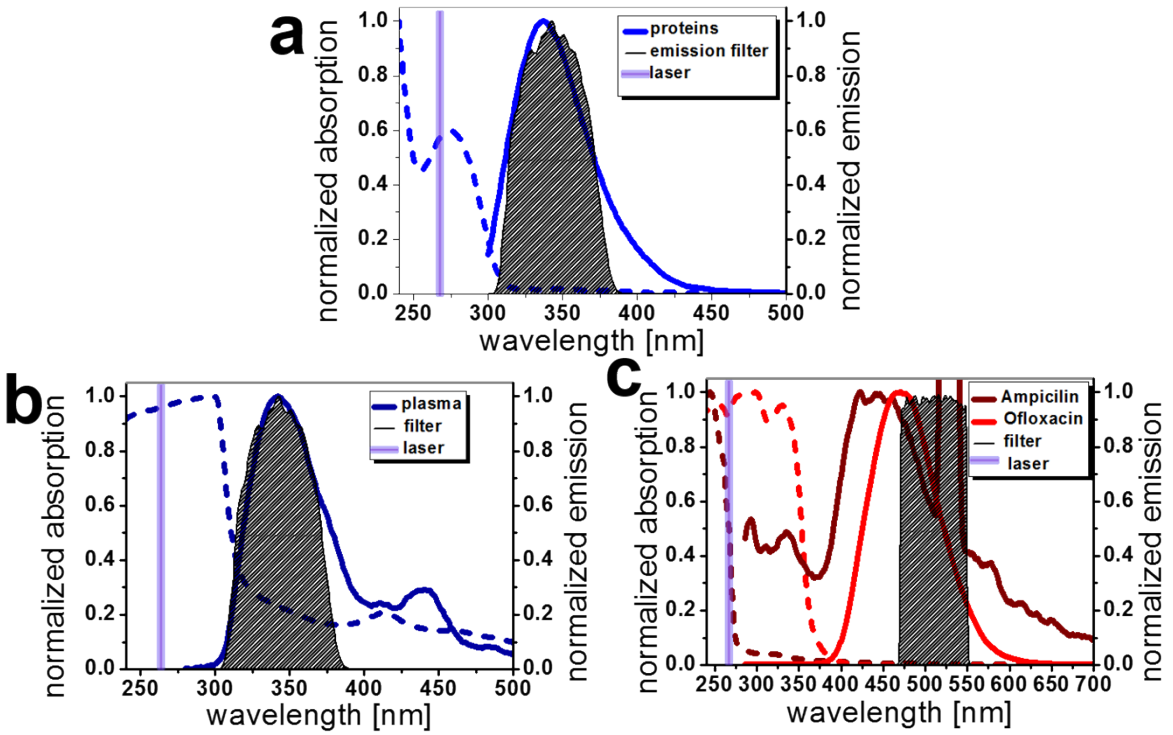

Figure S1. *Composite spectra of (a) the applied proteins α-lactalbumin and β-lactoglobulin B (dashed blue line: absorption, straight blue line: emission intensity by excitation at 266 nm, dark grey: emission filter, violet: laser), (b) bovine blood plasma (dashed dark blue line: absorption, straight dark blue line: emission intensity by excitation at 266 nm, dark grey: emission filter, violet: laser) and (c) the antibiotics ofloxacin and ampicillin (dashed red line: absorption of ofloxacin, straight red line: emission intensity of ofloxacin at 266 nm excitation, dashed brown line: absorption of ampicillin, straight brown line: emission intensity of ampicillin at 266 nm excitation, light grey: emission filter, violet: laser line).*

The fluorescence emission of ofloxacin was much brighter than that of ampicillin after excitation at 266 nm (Figure S1c) therefore we set the concentration of ampicillin around four times higher than the concentration of ofloxacin in the IEF for demonstration purposes.

S2.2. Characterization of the NIR fluorescent pH sensor

Absorbance and fluorescence of the NIR fluorescent sensor layer at pH 3 using 620 nm excitation (Figure S2) were recorded. At 266 nm excitation virtually no fluorescence of the pH sensor was detected.

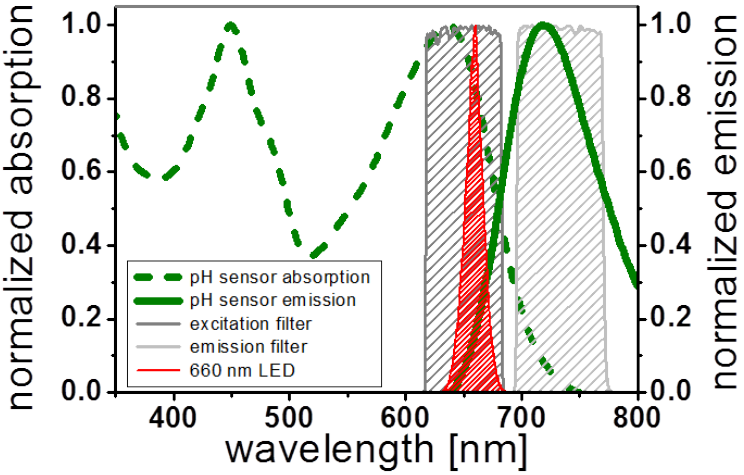

Figure S2. *Spectra of the pH sensor layer and the employed LED light source and filters (fluorescence of the pH sensor was excited at 620 nm and pH 3, dashed green line: absorption, straight green line: emission).*

The on-chip characterization of the sensor layer was performed via excitation with a 660 nm LED (M660L3, Thorlabs, 640 mW) which excites the NIR fluorescent pH sensor at the isosbestic point and 2.5x Fluor objective (NA 0.12, Zeiss, Jena, Germany) for Fig. S2a,c,d or a 20x UPlan FL N objective (Olympus, Hamburg, Germany) for the fast observation of response times in Fig. 2b. The pH sensitivity was examined with 10 mM BRBs from pH 2 to pH 10. The pK_a was determined to be 5.91 ± 0.09 (Figure S3a). Therefore the pH sensitive region extended from around pH 4.7 to pH 7.2. The response time of the pH sensor, which was measured with fast changes in the flow solution between pH 3 and pH 10 was determined to be a few seconds in both directions (t_{95} acidic → alkaline 5.9 s, t_{95} alkaline → acidic 7.1 s, Figure S3b). Because of the covalent linking of the indicator, the sensor layer showed a very high stability against a hydrodynamic flow at a flow rate of 25 µL·min^{-1} over 30 minutes with an intensity loss of 3.74 ppm·s^{-1} (Figure S3c). Applying constant LED illumination for 30 minutes, the intensities decrease with a rate of 26.8 ppm·s^{-1} (Figure S3d).

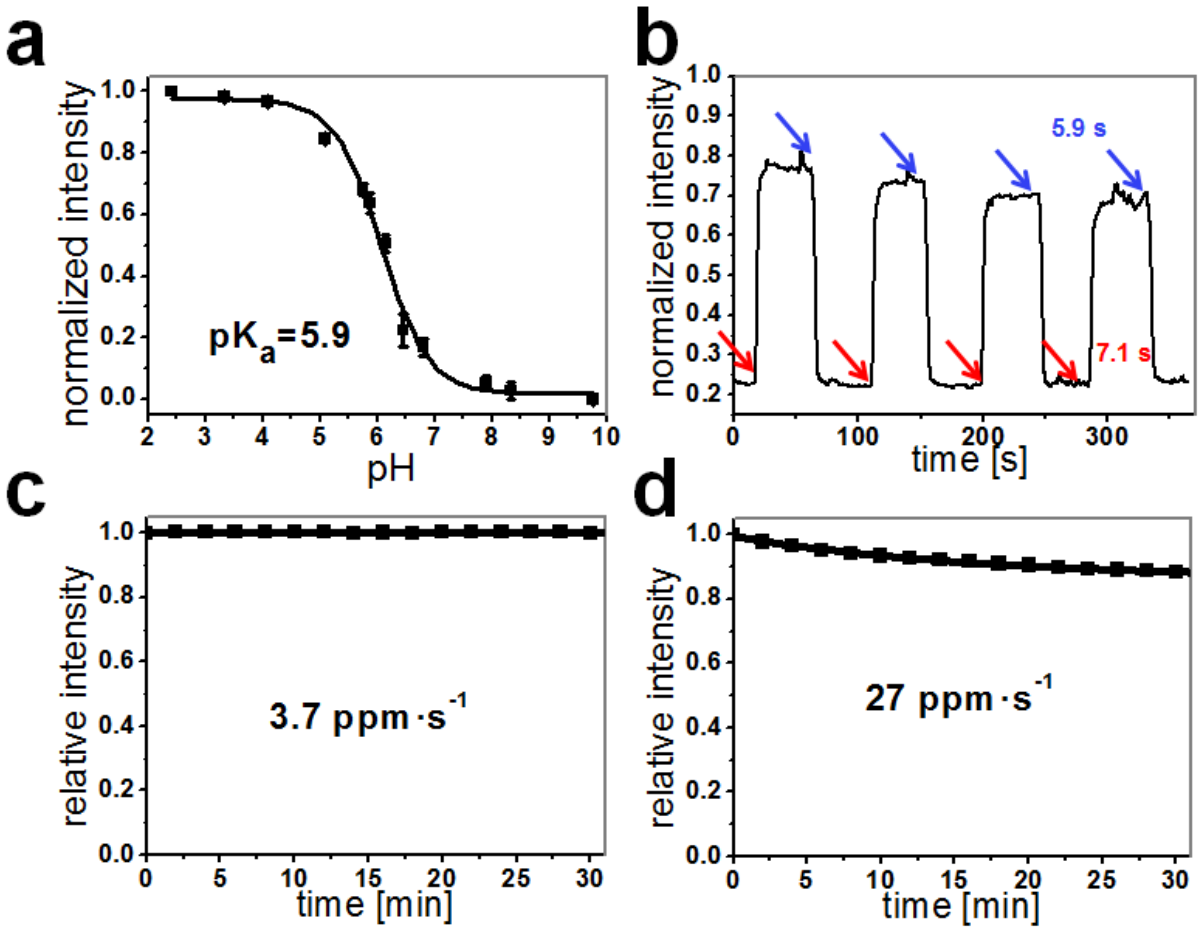

Figure S3. *NIR fluorescent pH sensor characterization via 660 nm LED excitation (1000 mA) at λ_{exc} 650/60 nm, λ_{em} 732/68 nm and a 2.5x microscope objective (a, c, d) or a 20x objective (b). (a) pH-dependency of the fluorescence intensity, (b) response time of the integrated sensor: red arrows: change to pH 3, blue arrows: change to pH 10, (c) flow stability of the sensor matrix at a flow rate of 0.45 mm·s^{-1}, (d) photostability at constant illumination over 30 minutes.*

S2.3. Characterization of the isoelectric focusing

To show that the separation of the analytes was caused by the isoelectric focussing and not by free-flow electrophoresis we took a macroscopic image of the separation of the antibiotics with a colour CCD Camera (Figure S4). The bands move almost perfectly parallel over several millimetres in the separation bed which is strongly indicative of a steady-state IEF. The parallel movement of the bands can also be seen in the supplementary video (SV1) that starts with switching on the electric field, shows the formation of the bands and then follows the bands along the separation bed to the outlet (SV1).

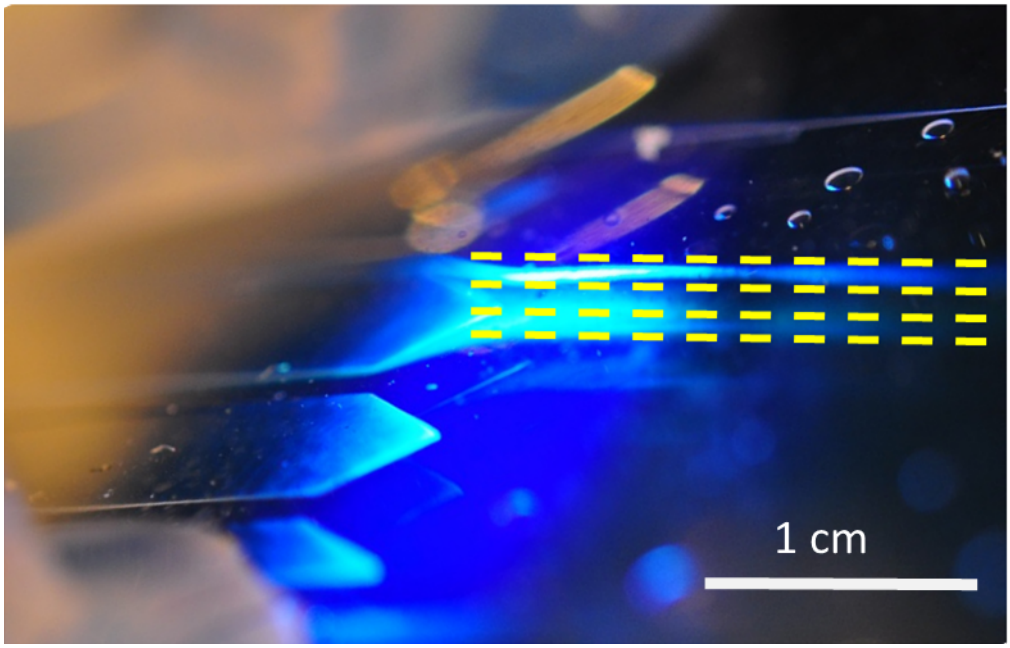

Figure S4. *Macroscopic colour CCD image of the antibiotics separation.*

S3. Mass spectrum analysis

Mass spectra were measured from the antibiotics mixture before the separation and from fractions obtained from different outlets using a 6520 Agilent Q-TOF mass spectrometer (Agilent, Santa Clara, USA) with an ESI source. ESI conditions were a temperature of 300 °C and a drying gas flow of 5 $L \cdot min^{-1}$. The nebulizer was set to 20 psig and the ionization voltage was 4000 V. The sample solution was pumped into the source with a flow of 10 $\mu L \cdot min^{-1}$ with a syringe pump PHD 22/2000 (Harvard Apparatus, Holliston, USA) and a glass syringe (Hamilton Gastight 1725, 250 µL, Hamilton, Bonaduz, Switzerland).

Table S1. Results of the mass spectra of different fractions collected at the outlets

sample	**determined m/z $[M+H]^+$**	**intensity (a.u.)**
before separation		
Ampicillin	350.117	279570
Ofloxacin	362.150	934277
Ampicillin fraction		
Ampicillin	350.115	37363
Ofloxacin	362.149	5620
Ofloxacin fraction		
Ampicillin	350.114	1512
Ofloxacin	362.150	225311

Supplementary References

1 D. Aigner, S. M. Borisov, P. Petritsch and I. Klimant, *Chem. Commun.*, 2013, **49**, 2139.