MICROCHIP FREE-FLOW ISOELECTRIC FOCUSING USING A PHOTOLITHIGRAPHICALLY INTEGRATED NIR FLUORESCENT pH SENSOR LAYER

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ABSTRACT

In this work, we show the fabrication, characterization and application of miniaturized free-flow isoelectric focusing platforms containing a covalently immobilized aminoperylene bisimide-based near infrared (NIR) fluorescent pH sensing layer obtained via photocopolymerization with acryloylmorpholine and oligoethyleneglycol diacrylate. These platforms were obtained via multistep photopolymerization and enable process observation as well as analyte identification via their isoelectric points during isoelectric focusing. These microchips were characterized and used for continuous separations of Atto 425 labeled proteins with in-line quantification of their isoelectric points.

KEYWORDS: micro free-flow electrophoresis, NIR fluorescent pH sensor, microchip isoelectric focusing, in-line analysis, isoelectric point quantification

INTRODUCTION

Recently we introduced a method for rapid determination of the isoelectric point (pI) of proteins and other (bio-)molecules using miniaturized free-flow isoelectric focusing (FFIEF) using a fluorescent pH sensor layer in the separation bed [1]. This assembly was able to determine the pI of proteins and other compounds on-chip in less than a minute with good precision and repeatability. Nevertheless the device still suffered from some drawbacks. Specifically, the readout in the visible region excluded the use of analyte labels in this spectral range, was subject to matrix background interference and the sensor was prone to photo bleaching. Novel near infrared fluorescent pH sensors based on 1-aminoperylene bisimides covalently grafted onto poly(acryloylmorpholine) were recently introduced [2]. They may be employed for spatially-resolved immobilization in microfluidic environments via photolithography.

THEORY

Free-flow electrophoresis (FFE) is a mild and continuous method for separation and preparation of biomolecules. In isoelectric focusing analytes are separated by their isoelectric point (the point of zero net charge, pI) in a pH gradient perpendicular to hydrodynamic buffer and analyte flows, typically established by ampholyte mixtures. The 1-aminoperylene bisimide derivative used herein displays a pH dependency of its fluorescence emission due to a photoinduced electron transfer (PET) from a piperazinyl moiety to the perylene ring causing fluorescence quenching. The PET is inhibited in acidic media when the amine is protonated. (Fig. 1a)

EXPERIMENTAL

A pH sensing layer (9.3 ± 1.6 µm thickness) consisting of 100 ppm (w/w) pH-sensitive NIR fluorescent aminoperylene bisimide **1** (Fig. 1a), 85.0 % (w/w) 4-acryloylmorpholine 14.8 % (w/w) oligoethyleneglycol diacrylate (OEG-DA) (M_n 700) and 0.2 % (w/w) 2-hydroxy-4'-(2-hydroxy)-2-methylpropiophenone as photoinitiator was photopolymerized on an acrylate-modified glass slide via UV illumination (365 nm) for 15 s. The microfluidic structure was photopolymerized on top of the sensor layer using OEG-DA (M_n 258) including 1 % (w/w) photoinitiator: 2,2-dimethoxy-2-phenylacetophenone (DMPA) and an acrylate-modified cover plate with holes for fluidic contacts. It was cured using a light impermeable mask and UV illumination with a 1.3 s exposure to form the channels and separation bed of the µFFIEF device. Before use the microchip was flushed with *iso*-propanol. The fabrication process of the microchips is schematically shown in Fig. 1.

Flow stability of the sensor layer was investigated using pH 3 Britton Robinson buffer (BRB) at a linear velocity of 1.48 mm*s^{-1} and the photo stability was examined via constant illumination with a 100 W Hg arc lamp through a 2.5x (NA 0.12) objective on an epifluorescence microscope setup. Response times were determined by alternate flow in the microchip with BRBs of pH 3 and 10 at 17.36 mm*s^{-1}.

For FFIEF separations seven inlets containing anolyte (BRB pH 3), catholyte (BRB pH 10), ampholytes (0.1 %, pH 4-7) and sample (Atto 425 labeled proteins) were used.

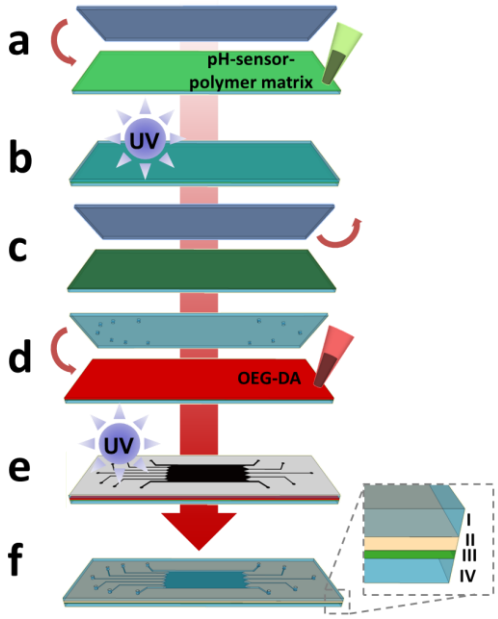


*Figure 1. Schematic illustration of fabrication of microfluidic free-flow isoelectric focusing chips with an integrated polymerized sensor layer: (a) deposition of prepolymer, (b) photopolymerization via UV illumination for 15 s, (c) removal of unmodified glass slide, (d) deposition of microstructure prepolymer (OEG-DA), (e) UV illumination for 1.3 s using photomask for microfluidic structure fabrication and (f) final microchip after removal of uncured prepolymer (**I** cover plate with inlet/outlet holes, **II** layer with microfluidic structure, **III** covalently bound sensor layer and **IV** bottom plate).*

RESULTS AND DISCUSSION

The chip-integrated sensor layer displayed a strong fluorescence and pronounced quenching of its fluorescence in the alkaline range via photoinduced electron transfer (PET). (Fig. 2a,b).

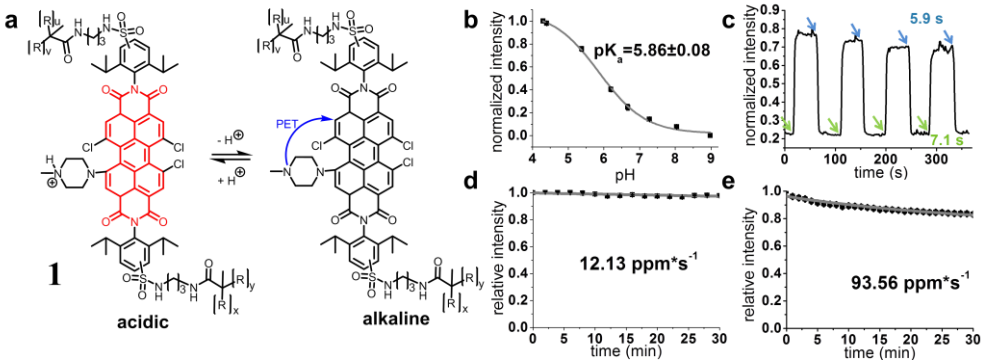


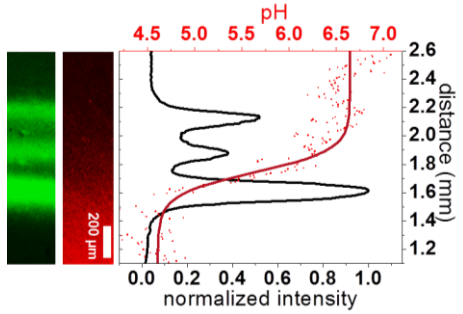
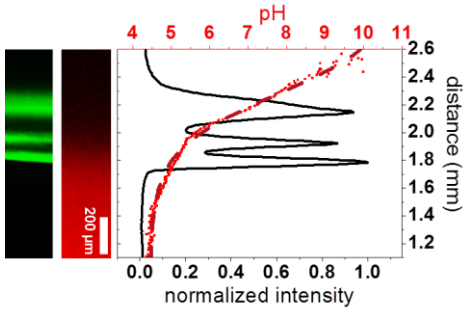
Figure 2: (a) Scheme of the PET resulting in pH sensitivity of this fluorophore, from [2]. Microchip-integrated sensor characterization : (b) pH response, (c) response times (pH change 3 ↔ 10), (d) flow stability (e) photostability.

The dynamic range of the chip-integrated pH sensor extends between a pH of around 4.0 to 8.0 (Fig. 2b). Response times t_{95} between pH 3 and 10 were 5.9 and 7.1 s, respectively. (Fig. 2c). The pH sensor matrix was found to be very stable towards flow represented by merely showing of 12.1 ppm fluorescence intensity loss per second at a linear velocity of 1.48 mm*s^{-1} (Fig. 2d), and displaying only moderate losses (93.6 ppm*s^{-1}) due to photo bleaching (Fig. 2e).

Two solutions containing protein mixtures (8 µM each) were separated on the novel µFFIEF platform. The first solution contained bovine serum albumin (BSA), conalbumin and myoglobin and the second lactoglobulin, lactalbumin and ubiquitin, all proteins were labeled with Atto 425. Simultaneous imaging of analyte separation and the pH sensing layer was achieved using distinct spectral channels on a microscope with CCD camera detection. In table 1 (Fig. 3 and 4) the false-colored fluorescence images of the separation of Atto425 labeled proteins and the sensor layer and the electropherograms of the

separation (black) with on-chip spatially resolved calculated pH (red, dashed) are presented. This IEF measurement shows a separation within a few micrometers. pIs were extracted and found to be comparable to literature values (table 1) for all separated proteins, confirming the applicability.

Table 1. Comparison of isoelectric points (pI) from the literature and observed pI during FFIEF.

Electropherogram with observed pH	proteins	literature pI	observed pI	deviation
 *Figure 3: µFFIEF of protein solution I*	myoglobin (horse heart)	6.5-7.2	6.63 ± 0.04	± 0.0
	conalbumin type I (chicken egg white)	6.0-6.3	6.34 ± 0.08	± 0.0
	bovine serum albumin	4.7-4.9	4.95 ± 0.08	+ 0.1
 *Figure 4: µFFIEF of protein solution II*	ubiquitin (bovine heart)	6.7	6.62 ± 0.04	-0.1
	β-lactoglobulin B (bovine milk)	5.2-5.4	5.40 ± 0.04	+0.0
	α-lactalbumin type III (bovine milk)	4.5-4.8	4.96 ± 0.03	+0.2

CONCLUSION

µFFIEF chips with integrated NIR fluorescent pH sensor layer were fabricated via photolithography and applied to proteins with on-chip near real-time detection of the pI of focused proteins. These devices enable essentially background-free spatially-resolved pH observation in the NIR and display good photo- and flow stability for IEF applications. In further research, upstream chip-integrated reactions or transformations with separation of products via FFIEF and on-chip pH monitoring could be realized or IEF of unlabeled proteins or other biomolecules with in-line process control.

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REFERENCES

[1] S. Jezierski, D. Belder, S. Nagl, "Microfluidic free-flow electrophoresis chips with an integrated fluorescent sensor layer for real time pH imaging in isoelectric focusing" *Chem. Commun*., 49, 904-906, 2013.

[2] D. Aigner, S. M. Borisov, P. Petritsch I. Klimant, "Novel near infra-red fluorescent pH sensors based on 1-aminoperylene bisimides covalently grafted onto poly(acryloylmorpholine)" *Chem. Commun*., 49, 2139—2141, 2013.

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