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Review

Micro free-flow isoelectric focusing with integrated optical pH sensors

Recently, a new observation method for monitoring of pH gradients in microfluidic free-flow electrophoresis has emerged. It is based on the use of chip-integrated fluorescent or luminescent micro sensor layers. These are able to monitor pH gradients in miniaturized separations in real time and spatially resolved; this is particularly useful in isoelectric focusing. Here these multifunctional microdevices that feature continuous separation, monitoring, and in some instances other functionalities, are reviewed. The employed microfabrication procedures to produce these devices are discussed and the different pH sensor matrices that were integrated and their applications in the separation of different types of biomolecules. The procedures for obtaining spatially resolved information about the separated molecules and the pH at the same time and different detection modalities to achieve this such as deep UV fluorescence as well as time-resolved referenced pH sensing and the integration of a precolumn labeling step into these platforms are also highlighted.

Keywords: Free-flow electrophoresis / In-line monitoring / Laboratory miniaturization and automation / Luminescent chemical sensors / Microfluidic chips

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1 Introduction

Electrophoresis is one of the most successful techniques in the field of microfluidics. Due to its attributes, particularly the need

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Abbreviations: BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BCECF-dextran, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein covalently bound to dextran nanoparticles; BRB, Britton-Robinson buffer; BSA, bovine serum albumin; CCD, charge-coupled device; DLR, dual lifetime referencing; ESI-MS, electrospray ionization mass spectrometry; FFE, free-flow electrophoresis; μ FFE, micro free-flow electrophoresis; FFIEF, free-flow isoelectric focusing; μ FFIEF, micro free-flow isoelectric focusing; FFZE, free-flow zone electrophoresis; FITC, fluorescein isothiocyanate; IEP, isoelectric point; HEMA, 2-hydroxyethyl methacrylate; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid; M_w , molecular weight; NIR, near infrared; OEG-DA, oligo ethylene glycol diacrylate; PAM, poly(acryloylmorpholine); PBI, N-(3-([N-(3-(methacryloylamino)-propyl)amino]sulfonyl)-2,6-diisopropyl-phenyl)-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 (a perylene bisimide compound); PET, photoinduced electron transfer; pHEMA, Poly(2-hydroxyethyl methacrylate); pI, isoelectric point; Ru(dpp)₃, Ruthenium (4,7-diphenyl-1,10-phenanthroline) dichloride; t-DLR, time domain dual lifetime referencing; TPM, (3-methacryloyloxypropyl)trichlorosilane

for a high surface to volume ratio, miniaturization leads to significant benefits in this separation technique, and microchip (capillary) electrophoresis has been investigated and demonstrated in countless variants and powerful platforms are now widely and commercially available [1, 2]. The development of other electrophoretic methods on microfluidic chips has been slower but led to interesting advances. Notable areas include microchip gel electrophoresis and micro free-flow electrophoresis.

Free-flow electrophoresis (FFE), also called carrier-free electrophoresis, is a powerful separation method that is capable of continuous spatial separation of a wide range of compounds from small inorganic or organic ions to large protein or nucleotide complexes or even organelles or whole cells [3, 4] and can be employed without denaturation of proteins and other biomolecules. However, it features only moderate separation efficiency. It is frequently applied for pre separation of complex biomedical samples and mixtures.

In recent years there has been much interest and many developments in miniaturized, often chip-based, FFE, termed μ FFE [5–7]. The idea is to combine the advantages of FFE in its continuous and preparative operation with the seamless process integration and better separation efficiency potentially afforded by miniaturized platforms on microfluidic chips.

Isoelectric focusing (IEF) is widely applied technique that is probably best known for often being one dimension of two-dimensional gel or capillary electrophoresis that are frequently employed for proteomics research [8–10]. There a pH gradient

is established in the separation area, usually via ampholyte mixtures (polymeric zwitterionic compounds). Substances bearing different charges are then migrating to their isoelectric point (pI or IEP), which is the pH where they bear zero net charge. IEF is used heavily in the free-flow format and the miniaturization of free-flow isoelectric focusing (FFIEF) onto microfluidic chips (micro free-flow isoelectric focusing, μ FFIEF) was demonstrated in several ways and formats [11–15 and references therein].

Microfluidic FFE platforms have been directly coupled with a number of distinct other on- or off-chip functionalities already. Cheng and Chang presented on-chip switchable pH actuators made of bipolar membranes preceding a μ FFE that allowed alternation between free-flow zone electrophoresis (FFZE) and FFIEF [15]. Jezierski et al. described a combined chip consisting of a reactor and a micro-FFZE for the separation of on-chip labeled amino acids [16]. Geiger et al. coupled a nano-liquid chromatography with a subsequent on-chip FFE [17], whereas Johnson and Bowser interfaced a CE separation directly with a microfluidic FFE [18]. Benz et al. presented a solution for coupling of μ FFE outlet streams with electrospray ionization mass spectrometry (ESI-MS) [19].

Despite these tremendous advances, the level of miniaturization present in modern micro-FFE devices leads to several challenges for chemical analysis and monitoring. An example is the determination of the isoelectric point in miniaturized FFIEF. Although the isoelectric point of zwitterionic molecules may be determined via several methods [20], IEF is a prominent procedure in this respect and directly applicable to compound mixtures. In macroscopic FFE the local pH may be measured via a suitable pH electrode directly at every outlet so that every fraction can be assigned a pI but this approach is poorly suited for miniaturized formats as there are typically fewer outlets and the channels are very small.

In order to quantify the pH gradient in IEF, in gel and capillary electrophoresis compound mixtures of defined pI's, so-called isoelectric point markers, are frequently employed. These are normally fluorescent organic dyes or labeled proteins. However, their use features several disadvantages, they are limited to distinct pI values, upon addition of these probes, samples and products in FFE are contaminated. And their pH (and consequently pI) resolution is limited by the underlying electrophoretic technique. Particularly for miniaturized FFIEF, a technique free of these shortcomings would be desirable.

The pH can be monitored spatially and with very high resolution on the macro-, meso- or microscale using fluorescent or luminescent pH sensor layers. These thin films with a height of typically a few micrometers have been applied for diverse applications in bioprocess monitoring and biotechnology, cell biology, medical and environmental research [21]. The integration of these pH sensors into microfluidic systems had been accomplished by diverse methods. Most of the devices were employed either for miniaturized cell culture [22–24] or enzymatic biosensing with pH transduction [25, 26]. Other work dealt with investigation of fluid dynamics [27, 28] or electrolysis [29].

Recently, several real-world biological applications of these pH sensors integrated into microfluidic chips have been presented, indicating a growing maturity of the field. Tahirbegi et al. used chip-integrated pH sensors, along with other sensors, for

the in-situ analysis of pesticides in tap water [30]. Gashti et al. employed a nanoparticle-based thin film to monitor changes at the attachment surface of a *Streptococcus salivarius* biofilm [31]. Gruber et al. used the read-out of chip-integrated pH sensors to improve the yield of enzyme microreactors [32].

In the last few years, my group and collaborators have introduced several different platforms for μ FFIEF that also contained integrated fluorescent pH sensor matrices and thus are able to monitor the pH and the pI of isoelectrically focused compounds almost in real-time. This is, in the view of the author, a substantial advance to competing methods such as the use of pI markers or *off-line* pH measurements at the outlet channels.

Since this method of pH sensing in μ FFE has, to the best of my knowledge, not yet been adapted, modified or extended by other research groups the review is based on work within my environment and gives a short overview of our efforts and implementations in this very young, but also very promising field. But hopefully, it can also serve to inspire others to use or develop related devices and methods for FFIEF or other separation methods.

2 Fluorescent pH sensing in isoelectric focusing

The first step in the IEF with integrated sensors is the fabrication of these devices. Unfortunately many established methods for luminescent sensor layer production are poorly compatible with microfluidic chip assembly and vice versa. But thanks to many advances in microfabrication the area has gained in pace tremendously in last decade and there is now a comprehensive toolkit available for integration as well as monitoring of these sensors in microfluidic devices [33]. Our efforts in the implementation of fluorescent pH sensing in FFIEF were greatly aided by earlier advances in microfluidic FFE chip fabrication and application. Inspired from work in which liquid oligo ethylene glycol diacrylate (OEG-DA) precursors were used to form microchannel networks and microvalves between two glass slides [34], a procedure for fabrication and application of μ FFE platforms using these materials was invented.

A short-chain OEG-DA (molecular weight (M_w) 258 Da), along with 1% (w/w) 2,2-dimethoxy-2-phenylacetophenone as the photoinitiator, were spread between two glass slides that were surface-modified with acrylate groups. A photomask with a μ FFE structure was applied and short near UV illumination caused polymerization of the microstructure between the glass slides, remaining unpolymerized OEG-DA was removed via access holes. A second polymerization with a longer chain OEG-DA (M_w 575 Da) and photoinitiator facilitated the integration of hydrogel walls separating the FFE bed from support channels. This resulted in fully functional μ FFE platforms within with a structural height of around 30 μ m and the microfluidic chips could be used for separation of organic dyes and labeled amino acids with separation performances comparable to other μ FFE platforms. In addition, the whole fabrication procedure was carried out in a regular chemical laboratory without the need for a dedicated cleanroom [35].

Figure 1. Manufacture of μ FFIEF chips with an integrated pH sensing layer. (A) Acrylated glass slide, (B) coating with OEG-DA hydrogel and UV exposure. (C) Spread of a short-chain OEG-DA prepolymer and (D) lid attachment, (E) UV exposure through photomask, (F) removal of uncured prepolymer, (G) photomask containing microfluidic structure, electrode channels are highlighted in gold. 1: anionic support channel, 2: ampholyte mixture, 3: sample solution, 4: cationic support channel, 5: cationic electrode channel, 6: anionic electrode channel, (H) fluorescence image of label channel. (I) fluorescence image of pH sensor channel. (J) Combined results of local pH (green) and label fluorescence (red). Arrows are indicating the direction of the hydrodynamic flow. Scale bars: 250 μ m. Adapted from [36] with permission from The Royal Society of Chemistry.

2.1 Microfluidic free-flow electrophoresis chips with an integrated fluorescent sensor layer for real time pH imaging in isoelectric focusing

PEG hydrogels polymerized from OEG-DA with a M_w of 575 Da, as described above, may be used as a matrix for pH sensing. Initial tests validated this functionality along with various pH probes. The main breakthrough in the manufacture of μ FFE chips with integrated pH sensors was the application of multiple photopolymerization steps with different OEG-DA precursors in order to create multiple layers. Because the polymer resulting from a short-chain OEG-DA precursor (M_w 258 Da) is dominated by polymerized acrylate groups and therefore hydrophobic, it does not mix with the PEG created from longer chain precursors. Therefore the microfluidic structure (from short-chain OEG-DA) may be manufactured right on top of a photopolymerized pH sensor matrix (from longer chain OEG-DA) using appropriate photomasks.

This led us to the following manufacturing cycle, displayed in Fig. 1 [36]. It starts with a standard borosilicate microscope glass slide that was acrylate-modified with (3-methacry

loyloxypropyl)trichlorosilane (TPM, Fig. 1A). In the next step a commercial pH probe, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) covalently bound to dextran nanoparticles (BCECF-dextran), is mixed with OEG-DA precursor (M_w 575 Da), photoinitiator and water. The coating mixture is spread manually as a thin layer between the bottom glass slide from the previous step and a non-acrylated top glass slide and illuminated for approx. 1 s with 365 nm light from a mercury arc lamp (Fig. 1B). This results in a stable pH sensor layer that is covalently bound to the acrylated bottom glass slide. The top glass slide is removed after the procedure.

Then a short-chain OEG-DA (M_w 258 Da) with photoinitiator is spread onto the glass slide containing the pH sensor layer (Fig. 1C) and an acrylated top glass slide is attached. The glass slide on the top contains inlet and outlet holes, that were powder blasted into the glass (Fig. 1D). Then a photomask with a suitable μ FFE structure is put on top of the assembly and again shortly illuminated with near UV light (Fig. 1E). Unpolymerized OEG-DA is removed using reduced pressure and the result is a finished μ FFE chip with an integrated pH sensor layer (Fig. 1F). After attachment of fluidic connectors and external electrodes to the

respective inlets and outlets the microfluidic chip may be used for electrophoretic separations. The fabrication of the assembly only takes a few hours.

If the procedure is applied as outlined above, it results in a pH sensor layer as well as a fluidic layer of approx. 20–25 μm thickness, respectively. The separation bed was 20 mm $\times$ 8 mm in length and width and the inlet and outlet channels were between 250 and 300 μm in width, flanking channels to which external electrodes were connected were 1 mm in width with a partitioning wall of 100 μm in width between the outer channels and the separation bed. The fluidic structure used for FFIEF is shown schematically in Fig. 1G: Inlet (i) contains an anionic support solution (20 mM NaOH), the inlets labeled (ii) the ampholyte mixture (2% Sigma ampholyte pH 3–10 with 0.1% (w/w) Tween-20), inlet (iii) the sample solution. Inlet (iv) is a cationic support solution (20 mM H_2SO_4), inlet (v) is the cationic electrode channel (a channel with an electrolyte solution connected to an external electrode, 20 mM H_2SO_4) and inlet (vi) the anionic electrode channel (20 mM NaOH). For attachment of external electrodes copper wires connected to a high voltage direct current source were pinched into a silicone tube and fixated with silicone glue.

The sensor layer covers the whole slide but is particularly visible in the areas where there are fluidic channels. The result is an overall homogeneous layer structure with very few visible defects. The pH sensing functionality was calibrated using Britton-Robinson buffers (BRBs) with ionic strengths and flow rates that matched the ampholyte solutions used for IEF in later experiments.

This pH probe shows features typical for fluorescein-based pH probes which are excited in the blue and emit in the green spectral range, the acid dissociation constant (pK_a) was determined as 6.23 with a dynamic range of around $\text{pK}_a \pm 2$. These values correspond well with known properties of this probe, therefore it can be concluded that the pH sensing functionality was successfully integrated into microfluidic FFE chips. Aside from sensing, the hydrogel-based bottom layer also serves as the electrical connection between the electrode channels and the separation bed.

These combined microfluidic chips were then used for the FFIEF of organic molecules and biomolecules. Sample, buffer and ampholyte solution were introduced into the chip and upon switching on the electrical field the formation of the pH gradient could be monitored with the pH sensor. Isoelectric focusing was carried out with small molecules and proteins.

As a first demonstration of protein analysis, the proteins bovine serum albumin (BSA) and conalbumin were separated. They were labeled with the pyrylium-based dye P503 which is advantageous for IEF because it does not change the charge of a protein upon labeling and also has a rather small molecular mass [37]. Therefore, in contrast to other labeling dyes, it is expected to influence the pI of proteins only insignificantly.

The literature pI of the two test proteins differs by around 1 pH unit (4.9 and 5.9, respectively). They could be separated using the above-mentioned solutions at 400 V and a linear velocity of 4.6 mm/s in the separation bed. A fluorescence micrograph of the separation (excited in the green, emission in the red spectral range) is displayed in Fig. 1H clearly showing the two focused protein bands. The pH was monitored at the same location but

in another spectral channel (excited in the blue, emission in the green spectral range, Fig. 1I) and the integrated results of both channels can be overlaid as in the graph in Fig. 1J showing the pH gradient in the FFIEF in green and the electrophoretic bands in red.

When the bands are focused, i.e. they are not migrating perpendicular to the flow direction in FFIEF anymore, the local pH corresponds to the pI of the molecule. This can be clearly observed in the separation shown here and the graph in Fig. 1J may then be used to determine the pI of the biomolecule simply by determination of the pH (green) of the center of the electrophoretic band (red). The results from multiple separations yielded a pI of 4.98 ± 0.41 for BSA and 5.95 ± 0.40 for conalbumin. This corresponds very well with literature values of 4.90 and 5.88, respectively.

It can therefore be concluded that by integration and application of a fluorescent pH sensor layer the local pH (and therefore the pI of zwitterionic molecules) in IEF can be monitored directly in the separation bed. This technology may be used to observe the formation and stability of the pH gradient in (FF)IEF as well as to determine isoelectric points of biomolecules in an ongoing separation within a few seconds. The determined pI may aid the assignment of a band to a particular compound. However, with the system implementation as described above there were still some weaknesses that we tried to improve in further work.

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

Aigner et al. have described novel pH sensor layers that are based on perylene bisimide (PBI) derivatives that were covalently grafted onto poly(acryloylmorpholine) (PAM) [38]. One attractive feature in the context of microfluidic chip fabrication is that they may be photopolymerized to a dedicated sensor layer. The probe molecule is copolymerized with acryloylmorpholine and OEG-DA yielding a polymer layer with a covalently attached pH probe. Importantly this prepolymer mixture may be integrated quite straightforwardly into a μ FFIEF chip manufacturing process using a procedure closely related to the pH sensor layer fabrication step outlined in 2.1. In our work we used 85.0% (w/w) acryloylmorpholine, 14.8% (w/w) OEG-DA (M_w 700 Da), 0.2% photoinitiator and 0.02% pH probe and illuminated the mixture in the near UV for 15 s.

The pH response of this probe is based on a photoinduced electron transfer (PET). Protonation under acidic conditions inhibits this PET and intramolecular charge transfer also contributes to the pH sensitivity that shows a pK_a of around 6. Importantly for the application in FFIEF, this pH probe shows absorption and emission maxima in the far red and near infra-red (NIR) part of the optical spectrum.

In our initial work on optical pH sensing in μ FFIEF discussed in 2.1. there was still some spectral crosstalk of the pH sensor fluorescence into the protein label fluorescence. The spectral position of this pH probe in the NIR as well as the straightforward photopolymerization thus made this perylene-based pH sensor

layer attractive for integration into our μ FFIEF platforms. To circumvent off-chip fluorescence labelling that causes a several fold increase in separation and analysis time as well as the required sample amount and also to increase the spectral separation we built a setup based on deep UV fluorescence. Deep UV excited fluorescence is capable of monitoring proteins, peptides and other biomolecules via excitation of aromatic residues such as tryptophan, tyrosine and other groups. It had previously been demonstrated to be useful for monitoring of unlabeled biomolecules in several variants of electrophoresis including μ FFE [39].

The optical setup was built around a standard inverted fluorescence microscope with free-space UV laser incoupling (Fig. 2A) and was able to accomplish several functions. A spectrally clean 266 nm excitation laser beam was collimated and expanded to approx. 200 mm² and guided to the microscope (Fig. 2B) via a custom top port in a transillumination configuration (Fig. 2C). The bottom side of the microfluidic chip as well as the (bottom side) microscope optical systems block all deep UV excitation light thereby acting as additional filters and the epi-illumination stage of the microscope is available for excitation of the pH sensor layer using a 660 nm light emitting diode (LED) and appropriate optics and filters [40].

Microfluidic chip fabrication was closely related to the procedure shown in Fig. 1, differences being the use of a quartz top glass slide for good deep UV transmittance and the use of spacers for the microfluidic network resulting in structural heights of around 10 μ m for the pH sensor layer and 70 μ m for the microfluidic structure.

The application of these platforms was then demonstrated in the FFIEF of various biomolecular mixtures. We demonstrated

the FFIEF of the unlabeled proteins lactalbumin and lactoglobulin, the separation of (unlabeled) blood plasma into its albumin and globulin fraction and the IEF of the antibiotics ampicillin and ofloxacin. The integrated pH sensor layer could be used for monitoring of the pH gradient and determination of the pI of the biomolecules (or fractions in the case of the plasma separation) and the results were in good agreement with literature values enabling the assignment of the individual peaks.

In this study we also collected fractions at the outlets for the antibiotics separation and analyzed them via ESI-MS. The MS data confirmed the successful separation of the compounds with only a very small residue left of the respective other compound in each fraction and the ability of the platforms to transport the separated compounds to different locations [40].

2.3 Rapid isoelectric point determination in a miniaturized preparative separation using jet-dispensed optical pH sensors and micro free-flow electrophoresis

The platforms discussed in 2.1 and 2.2. were able to record the pH gradient and pI's in μ FFIEF and performed quite well. However, they are limited to photopolymerization-based polymer matrices for use as pH sensor layers, which is a severe limitation since this excludes many pH sensor layers that have proven to be very useful. In order to expand the capabilities we have devised a method that is able to integrate inkjet-printed pH sensor layers into μ FFIEF platforms [41]. To this end we developed an appropriate sensor chemistry and fabrication procedure.

Figure 2. (A) Schematic illustration of the employed measurement setup with deep UV excitation. (B) Image of the microfluidic chip on an inverted fluorescence microscope with laser excitation from above and, (C) macroscopic images of the excitation laser light path. Adapted from [40] with permission from The Royal Society of Chemistry.

pH probes covalently bound to Poly(2-hydroxyethyl methacrylate) (pHEMA) were synthesized using a combination of previously described protocols. Fluorescein in its activated isothiocyanate form (FITC) was coupled directly to pHEMA using a *N,N*-dimethylacetamide linker whereas 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was coupled to an amino-modified pHEMA-based copolymer.

Fluorescent pH sensor layers from these polymers were inkjet-printed on acrylated glass slides from 5% (w/w) ethanol/water or dimethylformamide (DMF) solutions with the help of a 100 μm inner diameter printhead at a robotic station at 70°C glass slide temperature. This procedure could print pH sensor spots or rows (and potentially other shapes) with a diameter of 300 to 600 μm and thicknesses of 0.4 to 2.4 μm with high spatial accuracy.

The integration of these glass slides with inkjet-printed pH sensor matrices into functional μFFIEF platforms is schematically shown in Fig. 3. The short-chain OEG-DA prepolymer (M_w 258 Da) for fabrication of the microfluidic structure and photoinitiator are spread between an acrylated and a plain glass slide (Fig. 3A, B) and photopolymerized (Fig. 3C, D). After removal of OEG-DA residue (Fig. 3E) the plain glass slide is removed (Fig. 3F) and replaced with a slide containing an inkjet-printed pH sensor structure (Fig. 3G). Using small amounts of OEG-DA and photoinitiator on the edges of the printed slide it can be bound to the assembly via another photopolymerization (Fig. 3H) and form a functional μFFIEF chip (Fig. 3I, J). Fig 3K and 3L shows real-color fluorescence images of pH sensor rows and spots integrated into FFIEF platforms.

The strength of this approach is in the ability to create sensor areas of flexible geometry and low thickness down the sub- μm regime. This results in very fast response times, e.g. for the FITC-pHEMA layer down to 300 ms. Different pH probes, each with their own characteristics, can be employed with this procedure.

The fluorescein-based probe employed here had a pK_a of about 7 whereas the HPTS-based probe had a pK_a of about 9 and is better suited for the alkaline range.

The sensors were employed for separation of proteins in both a sensor array and a sensor row format. Both approaches performed well in being able to monitor the pH gradient in IEF and allowed determination of the pI of focused bands with mostly only small deviations to literature values. However, some proteins, that had a pI outside of the dynamic range of the sensor matrix showed a larger deviation. The sensor rows proved a little bit better suited for spatially resolved pH determination than the sensor spots since they allowed pH determination all over the separation bed whereas the sensor spots were limited to their respective locations. However, sensor arrays are potentially better capable of incorporating multiple different pH sensors.

For comparison five isoelectric point markers, molecules with known pI 's from 4.0 to 9.0, were focused and the results compared with the visual readout of the markers alone. It was clear that the integrated pH sensor gave a much more detailed and better-resolved readout of the pH gradient in the microchip but the dynamic pH range is a bit less than that of multiple markers (here 5 pH units). In the future, chip integration of multiple optical pH sensors could help to extend their dynamic range.

2.4 Development of microscopic time-domain dual lifetime referencing luminescence detection for pH monitoring in microfluidic free-flow isoelectric focusing

In the works discussed so far microscopic fluorescence intensity-based readout was performed in a spectral channel for the analyte

Figure 3. Fabrication of μFFIEF chips with inkjet-printed pH sensors, schematic illustration. (A) Silanized cover glass plate and non-silanized bottom glass slide, (B) deposition of OEG-DA prepolymer including photoinitiator between the slides, (C) alignment and (D) UV exposure with photomask. (E) removal of non-polymerized residue, (F) removal of the bottom plate. (G) Attachment of a glass slide containing the inkjet-printed sensor structure, (H) UV exposure and (I) schematic of a completed FFIEF chip with sensor rows. (J) A real-color fluorescence image of the resulting microfluidic chip with UV excitation, (K) and (L) fluorescence micrographs of pH sensor rows and spots, respectively. On the left hand side, a schematic cross-section through the microchip assembly is displayed. Reprinted with permission from [41]. Copyright 2014 American Chemical Society.

and another one for the pH sensor, respectively. When the goal is mainly tracing the separated compounds as in the analyte channel, this approach typically gives sufficient data quality. However, for quantitative evaluation, as required in the pH sensor channel, intensity-based imaging is prone to some shortcomings. Its accuracy is compromised by spatially inhomogeneous excitation and detection efficiency, leaching and bleaching in the hydrogel membrane matrix. Furthermore, intensity-based sensing needs a very homogeneous indicator distribution or extensive calibration. Alternative, referenced, sensing approaches are of considerable potential to improve the precision and accuracy of pH monitoring in FFIEF.

For many applications, ratiometric fluorescent pH sensors were developed either based on a wavelength-ratiometric approach using dual excitation or emission chromophores or different spectral behavior of the fluorescent probe and a reference. But this needs another spectral channel, and some background cannot be suppressed by this method. Luminescence lifetime-based schemes are favorable for chemical sensing, because they eliminate many interferences. One very successful pH monitoring technique is dual lifetime referencing (DLR) in the time-domain (t-DLR) that uses the lifetime contrast between a short-lifetime pH responsive probe and a long lifetime inert reference [42].

We adapted this method for the pH observation in μ FFIEF. An instrumental setup was developed based on an inverted optical microscope with electronic synchronization of an LED light source and a modulated charge-coupled device (CCD) camera. In addition, probes were developed and modified that allowed the implementation of t-DLR within FFIEF platforms. A pH sensing matrix was formed in a procedure related to 2.3, utilizing amino-modified pHEMA,

formed from 2-hydroxyethyl methacrylate (HEMA) and N-(3-aminopropyl)-2-methylacrylamide (Fig. 4A). This copolymer was reacted with the activated pH probe 5(6)-carboxyfluorescein-N-hydroxysuccinimyl-ester to form a fluorescent and pH-sensitive hydrogel matrix. As non-pH sensitive reference with a long luminescence lifetime ruthenium-4,7-diphenyl-1,10-phenanthroline dichloride ($\text{Ru}(\text{dpp})_3$)-doped polyacrylonitrile (PAN) nanoparticles were employed, which were created in a nanoprecipitation procedure with some modifications, based on the procedure in [43].

The carboxyfluorescein-pHEMA and the $\text{Ru}(\text{dpp})_3$ -PAN were mixed and inkjet-printed as 370 μm wide and 400 nm thick sensor rows on glass from ethanol/water dispersions as discussed before. The microfluidic structure in this work was generated via laser plotting of acrylate-based adhesive foil, which was brought between acrylate-modified glass slides. One of the slides contained an inkjet-printed pH sensor layer and in the end the assembly was fixed via application of a pressure of 10 kN.

This integrated pH layer was calibrated, showed a dynamic range of pH 4 to 8 and a very fast response of under 0.5 seconds. The microfluidic chip was then used for the FFIEF of a labeled protein mixture containing β -lactoglobulin A, conalbumin and myoglobin. The proteins could be separated with chromatographic resolutions from 0.9 to 1.3 and their pI could be determined with 0.2 pH units precision [44]. The results corresponded well with literature values. A remaining challenge was identified in the fast photobleaching particularly of the carboxyfluorescein in the hydrogel which allowed the illumination use of this matrix without recalibration only for a short time. The integration of pH probes with better photostability in this system is to be explored in the future.

Figure 4. Preparation of the sensing matrix for t-DLR-based pH imaging in FFIEF. (A) Synthesis of an amino-modified pHEMA hydrogel, (B) covalent attachment of carboxyfluorescein NHS-ester to amino-modified pHEMA. (C) Preparation of PAN nanoparticles doped with $\text{Ru}(\text{dpp})_3$. (D) Real color image of a dispersion of the carboxyfluorescein-pHEMA hydrogel prepared in (B). (E) Real color image of a dispersion of the $\text{Ru}(\text{dpp})_3$ -PAN nanoparticles prepared in (C). (F) Absorbance (dashed) and emission (solid line) spectra of carboxyfluorescein-pHEMA (green) and $\text{Ru}(\text{dpp})_3$ -PAN nanoparticles (orange). Excitation (red) and emission (blue) filters and the excitation light LED (blue, filled) spectra are also displayed. Reprinted with permission from [44]. Copyright 2015 John Wiley & Sons, Inc.

2.5 Continuous on-chip fluorescence labelling, free-flow isoelectric focusing and marker-free isoelectric point determination of proteins and peptides

The preceding platforms allowed the FFIEF with integrated pH sensing using different fabrication and detection methodologies. In the work highlighted in 2.1, 2.3 and 2.4 the biomolecules were labeled *off-chip* in a reaction vial and purified via size exclusion chromatography. This is an additional, elaborate, preparation step that lengthens the overall procedure significantly and typically needs more biological sample amount than the μ FFIEF. In 2.2 a viable alternative was introduced employing the intrinsic UV fluorescence of some proteins and biomolecules. However, this approach is not universal since many compounds do not show significant UV fluorescence, and furthermore needs an extensive and expensive optical setup and is of lower sensitivity than label-based imaging in the visible range.

This led us to produce an FFIEF chip, which is essentially a close relative of the one discussed in 2.2. We employed the same dual photopolymerization procedure to create a pH sensor layer, made from a NIR-emitting PBI derivative covalently grafted onto a PAM-PEG copolymer, and on top of it the microfluidic structure from a polymerized short chain OEG-DA. Because in this work we did not use UV fluorescence, borosilicate glass slides instead of fused silica ones were employed for chip fabrication. The only design modification was that the middle straight channel inlet for the biomolecule sample was replaced by a serpentine channel reactor structure of 50 mm effective length that led directly to the μ FFIEF. This, however, allowed the implementation of an on-chip precolumn labeling procedure prior to the FFIEF. Thereby this microfluidic chip could be employed for flow fluorescence labeling, FFIEF with integrated pH sensing as well as pI determination at the same time [45].

The labeling procedure was carried out with the coumarin label Atto 425-NHS ester that was introduced in one inlet in pH 9 labeling buffer with some dimethylsulfoxide (DMSO). In the other inlet biomolecules were dissolved in 1% ampholyte solution and diluted in the same labeling buffer. The flow reaction

was optimized for each separation, good results could be obtained up to a flow velocity of around 0.5 mm/s or ca. 2 min total labeling time. The capability of the assembly for subsequent labeling, focusing and pH monitoring could be demonstrated using sample solutions containing either one, two or three proteins, lactalbumin, lactoglobulin and myoglobin. After labeling and IEF a corresponding number of bands could be observed in the Atto 425 spectral channel. The integrated pH sensor allowed monitoring of the pH gradient and determination of the pI of focused molecules.

On-chip labeling and IEF was also performed with the neuropeptides endothelin, oxytocin, leucine-enkephalin and neurotensin that can be clearly resolved and their pIs determined by the integrated sensor (Fig. 5A). The separation of the aforementioned, on-chip labelled, proteins is displayed in Fig. 5B. In both separations, compounds with a pI difference of less than 1 could be clearly resolved. Lastly, a tryptic digest of the peptide physalaemin was reacted and isoelectrically focused. The digest was executed off-chip and then the products were labeled, separated and detected with this chip assembly (Fig. 5C). This nicely demonstrates the versatility of this chip platform.

3 Concluding remarks

Micro free-flow isoelectric focusing with integrated optical pH sensors is an integrated chip technology that combines advances in the fields of microfabrication, miniaturized separations, and chemical sensing. The generation of these platforms had been accomplished with techniques from the areas of photolithography, inkjet printing and laser cutting. Different pH sensor chemistries were adapted for these devices and introduced via photopolymerization or microspotting. These integrated pH sensors allow the monitoring of the pH gradient in FFIEF almost in real time and may be used to determine the pI of focused bands, which in turn, aids peak assignment and molecular property determinations.

These platforms were used in the isoelectric focusing of proteins, peptides, antibiotics, plasma and other compounds. Even

Figure 5. IEF with integrated pH monitoring of on-chip labeled compounds. pH channel (red) and label fluorescence (green) are overlaid in the graphs at the bottom. (A) μ FFIEF of the peptides endothelin (En), oxytocin (Ox), leucine-enkephalin (LE) and neurotensin (Ne) with integrated pH monitoring, (B) Isoelectric focusing of the proteins lactalbumin (La), lactoglobulin (Lg) and myoglobin (My), (C) separation of the products from a tryptic digest of physalaemin. Adapted from [45] with permission from The Royal Society of Chemistry.

unlabeled biomolecular mixtures can be separated via their detection in the deep UV or precolumn on-chip labeling. However, so far only few works exist in this area and even micro-FFIEF itself is still a rather small and young field with much growth potential. Future work will improve analytical performance, information content (e.g. via the use of multiple sensors) or fabrication procedures and see the adaption of these techniques to other separation methods or other miniaturized systems.

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