Novel pH indicator dyes for array preparation *via* NHS ester activation or solid-phase organic synthesis

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We present two ways of fast and easy immobilisation of a naphthalimide chromophore with a pH-sensor function. The immobilised dyes exhibit absorbance and emission in the visible spectral range, large Stokes' shift, fluorescence properties that are comparable to their water-soluble form, and full reversibility in pH response

Introduction

Array technologies provide a progressive platform for multiparameter analysis and are widely used in genomics and proteomics. Main advantages are the massive parallel readout of information using a common readout technique for all of the integrated spots. Recent developments of array technologies focus on the application in metabolomics and parallel process analysis. Furthermore, there is an increasing demand to use arrays for the verification of quality and identity of complex mixtures from natural sources *e.g.* in food industry and cosmetics. Therefore, sensor molecules that combine a receptor functionality for analyte recognition with a sensing substructure and an interface for covalent immobilisation on a suitable planar support are required.

Fluorophores that can be successfully applied are based on a single type of fluorophore structure, common to all integrated sensor molecules. Signal transduction is mediated by interaction of the receptor with the fluorophore in a way, that binding of a specific analyte at the receptor substructure results in a change of fluorescence properties *e.g.* quantum yield or frequency shift of the emitted light.

Naphthalimides have been reported to satisfy these requirements due to their excellent stability and the large variability of side chains for implementation of different receptors, that interact *via* photoinduced electron transfer with the fluorophore as well as the introduction of immobilisation interfaces *e.g.* carboxyl or hydroxyl groups. Naphthalimide derivatives are approved molecules for optical sensors, for non-doping OLEDS and laser dyes.[1,2] Functionalised naphthalimide chromophores have been under examination in solution as sensor dyes for analytes such as mercury ions,[3,4] zinc ions,[5] saccharides[6,7] and for pH-changes.[8,9]

The next step towards application is the covalent immobilisation of such sensor dyes, because it prevents leakage of the dye from the sensor membrane, Niu *et al.* developed an immobilised methylpiperazinyl derivative of naphthalimide[10] using the principle of photopolymerisation.

The main disadvantage of these approaches is the incompatibility with spotting techniques that are used for array preparation. In this work hydrogel thin films of *O*,*O*′-bis-(2-acryloylamidopropyl) polyethylene glycol 1900 (PEGA), a material primarily developed as a support for solid-phase organic synthesis (SPOS)[11,12] comprising amino groups, were used as a support for the sensor dye. Covalent immobilisation was performed by coupling of the *N*-hydroxysuccinimidyl ester of the sensor molecules to the reactive thin film, so that sensor molecules can be immobilised locally by the use of spotting techniques.[13] Alternatively, sensor molecules can be directly immobilised by SPOS to obtain homogeneous layers and to show the chemical stability of the system.

Here we report on methods for the immobilisation of pH sensor dyes using array technology. The spectra of the immobilised pH-sensitive dyes were characterised in comparison to their water-soluble form to evaluate effects of immobilisation on the performance.

Results and discussion

Generally, for the first step of synthesis 4-bromo-1,8-naphthalic acid anhydride was used.[14] For structure **1** this was reacted with 6-aminohexanoic acid and then the intermediate was reacted with 1-methylpiperazine.

Structure **1** also was used for the synthesis of **2** by immobilising it to the hydrogel matrix *via* amide bond after NHS ester activation. Solid-phase organic synthesis was used for the preparation of structure **3**, therefore 4-bromo-1,8-naphthalic acid anhydride was connected directly to the amino-functionalised hydrogel matrix *via* imide bond. In the second step this intermediate was reacted with 1-methylpiperazine to give compound **3** as an immobilised pH-sensor dye (Fig. 1)

The fluorescence properties of the sensing system were first evaluated with aqueous solutions of **1**. For this, **1** was dissolved in buffer solutions pH 5 to 9, and increases in signal intensity at the maximum of emission at 530 nm were found when the pH was decreased (Fig. 2). Accordingly, the fluorescence quantum yields were determined with $\Phi = 2.4\%$ at pH = 9.0 and $\Phi = 40.0\%$ at pH = 5.0 for solutions of **1**.

The signal increase is attributed to the modulation of the intramolecular photoinduced electron transfer from the nitrogen lone electron pair to the naphthalimide fluorophore caused by protonation.[15–18]

Sensor layers **2** and **3** were fixed in a flow cell and characterised by exposure to buffered solutions while the signal at the maximum of emission was detected during this process. After a short conditioning time (1 h) with increasing fluorescence intensity

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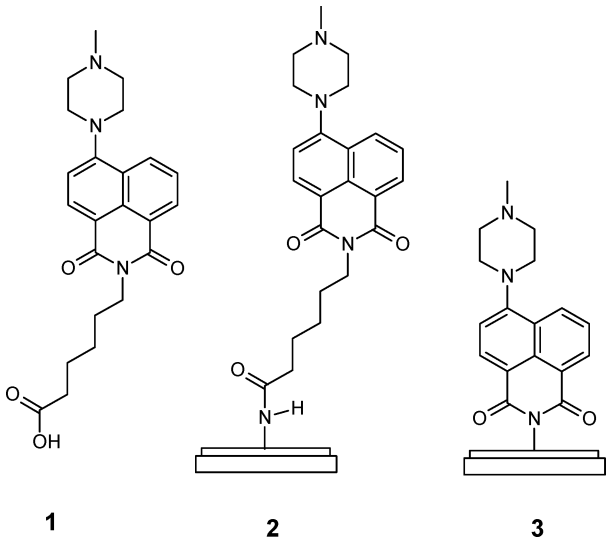

Fig. 1 Water-soluble compound **1**, immobilised *via* amide-bond (**2**) or solid-phase-synthesis (**3**).

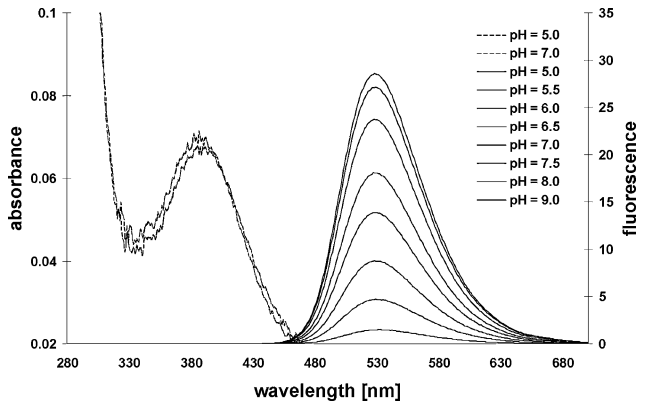

Fig. 2 Fluorescence spectra of compound **1** in solution of phosphate buffers at different pH, $\lambda_{exc} = 400$ nm.

caused by the swelling of the hydrogel-matrix a stable signal was obtained. Using the same excitation wavelength $\lambda_{exc} = 400$ nm as for **1**, the emission maximum of **2** and **3** was found at 522 nm. This shift to shorter wavelengths possibly is caused by the lower polarity of the polymeric microenvironment (comparable to dioxan/water 1:1) compared to the plain aqueous solution (Figs. 3 and 4). No

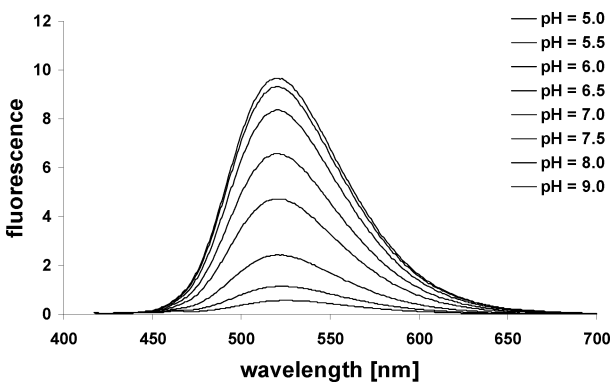

Fig. 3 Fluorescence intensity of substrate **2**, measured in a flow-cell with phosphate buffers at different pH, $\lambda_{exc} = 400$ nm.

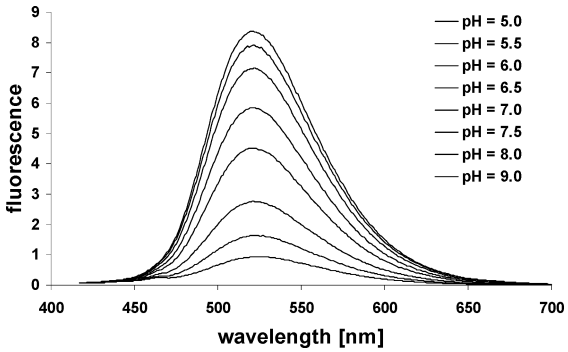

Fig. 4 Fluorescence spectra of substrate **3**, measured in a flow-cell with phosphate buffers at different pH, $\lambda_{exc} = 400$ nm.

leaching of the dyes was observed during a period of several hours (see also Fig. 6).

The pH-sensitivity of the immobilised dyes was evaluated by flushing the layers with different buffer solutions from pH 5.0 to 9.0 in the flow-cell. In comparison to **1** the layers **2** and **3** showed similar changes in emission spectra with changing the pH-values. Thus, lowering the pH-value from 9 to 5 also caused signal increases in emission.

The pK-values of the dissolved and immobilised sensor dyes were determined from the spectra and found to be 6.86 for **1**, 6.85 for **2** and 6.89 for **3** (Fig. 5). So the changes in the polarity of the matrix exhibit only a minor effect on the protonation of the dye. According to the Henderson–Hasselbalch equation the sensitivity range of **1**, **2** and **3** was found to be 3 pH units.

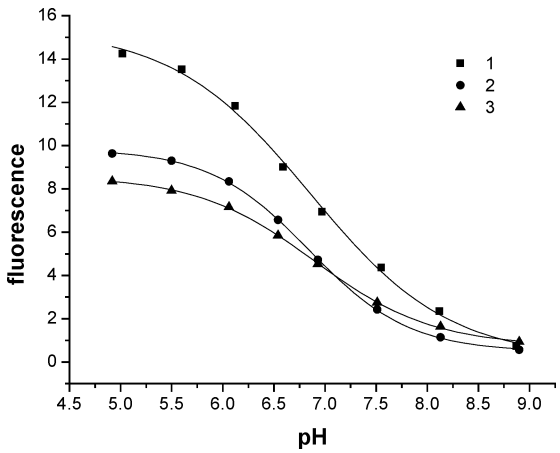

Fig. 5 Fluorescence intensity *vs.* pH of **1**, **2** and **3** measured at the maximum of their emission spectra, sigmoidal fit for pK-values.

Finally the sensor reversibility was evaluated. Therefore, the layers **2** and **3** were flushed with buffered solutions of pH-values 3.0, 7.0 and 9.0 and timedrive spectra were measured.

According to Fig. 6a and b, a fully reversible response of the pH-sensing system was found. The changes in intensity of **2** and **3** are comparable to **1**. The response time was faster in changing to stronger acidic or basic pH (5–20 s), but slower in the region of neutral buffer conditions, around the pK-value of the dyes (60–120 s).

Binding our dyes to the hydrogel *via* an amide bond opens the possibility to selectively immobilise sensor dyes by using

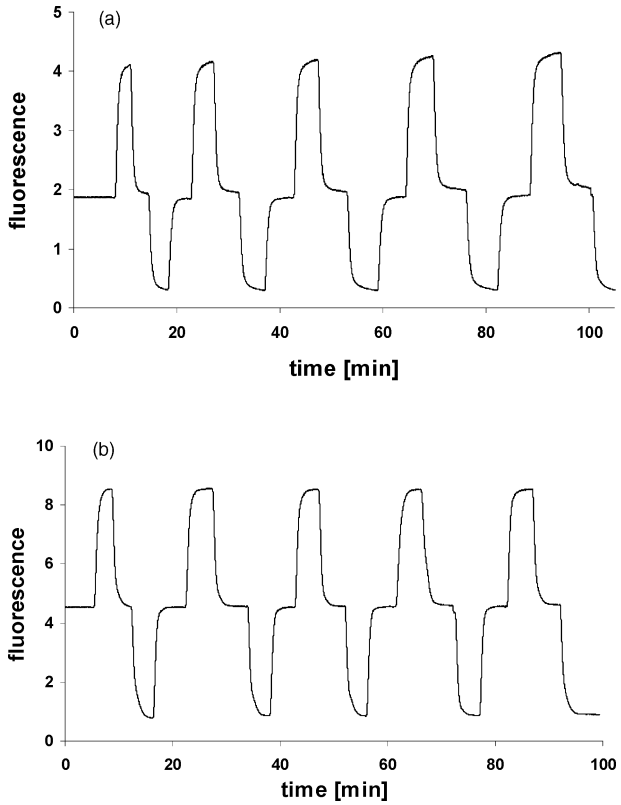

Fig. 6 Signal reproducibility of **2** (a) and **3** (b) while flushing with buffered solutions of pH 3, 7 and 9 in a flow-cell, $\lambda_{exc} = 400$ nm.

spotting techniques. Accordingly, hydrogel–sensor-dye systems and hydrogel–reference-dye systems were spotted on glass plates. The spots were measured with READER, a fluorescence reader that was developed by the company Sensovation, pictures were taken by a CCD-camera (coolSamBa HR-320) from Sensovation as well. In our case a morpholinium derivative, similar to **1** but without the pH-sensitive nitrogen, was used as the reference dye **4** (Fig. 7).

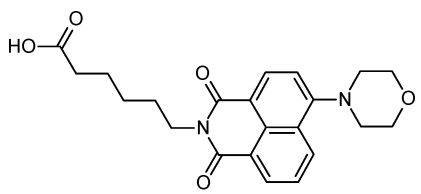

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Fig. 7 Reference dye **4**, optical properties comparable to **1**, **2** and **3**, but without pH-sensitivity.

The CCD pictures show the changes in emission of the spots based on the pH indicator while the emission of the reference remained stable with changing pH-values (Fig. 8). Similar to the pH-dependent fluorescence measurements of **1**, **2** and **3**, an increase in signal intensity was found with decreasing pH-value for the spots of **2**.

In summary, we found a way to easily immobilise fluorescent pH-sensitive dyes that show significant and reversible signal changes in contact with buffered solutions from 5.5 to 8.5 in

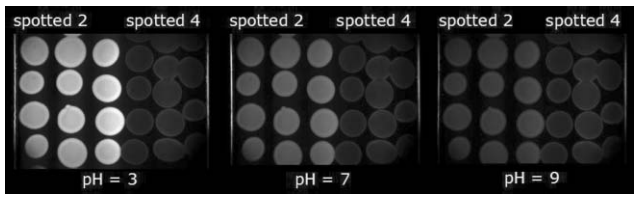

Fig. 8 CCD picture of hydrogel with spotted **2** and spots with reference dye **4** at different pH values.

reversible reactions. Furthermore, structure **3** was prepared under harsh conditions in SPOS and because of this it was possible to show the high stability of our hydrogel-film. Furthermore **2** was synthesised as a first step of miniaturisation of the sensing system for applications in arrays. In the near future we will extend this approach to new naphthalimide-based sensor dyes for biomolecules.

Experimental

General

The fluorescence spectra were recorded on a Spex Fluorolog 3 (Jobin Yvon) spectrometer, while absorbance spectra were recorded on a Lambda 16 UV-VIS spectrometer (Perkin Elmer), both at 25 ± 2 °C. The measurements at different pH-values (5.0–9.0) were performed by using a 67 mM Sørensen's phosphate buffer.

Synthesis of 6-(6-(4-methylpiperazin-1-yl)-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl) hexanoic acid 1

4-Bromo-1,8-naphthalic acid anhydride was suspended in ethanol, the suspension was warmed to obtain a clear solution and then an equimolar amount of 1-aminohexanoic acid was added to the solution. After refluxing for 5 hours the white solid was washed with ethanol and then recrystallized from ethanol to give 6-(6-bromo-1,3-dioxo-1*H*-benzo[*de*] isoquinolin-2(3*H*)-yl) hexanoic acid. In the second step this intermediate was refluxed for 10 hours with equimolar amounts of 1-methyl-piperazine in 2-methoxy-ethanol. This product was purified on silica gel using dichloromethane/methanol 5:1 to give **1** as a yellow solid. Yield: 80%. UV absorption: $\lambda_{max}(H_2O)$/nm: 380. ^{1}H NMR: δ_H (250 MHz; MeOD; Me_4Si): 1.45 (m, 2 H, -CH_2); 1.67 (m, 2 H, -CH_2); 1.67 (m, 2 H, -CH_2); 2.23 (m, 2 H, -CH_2); 2.36 (s, 1 H, CH_3); 2.62 (t, 2 H, N-CH_2-aryl); 3.13 (t, 2 H, N-CH_2-aryl); 4.1 (t, 2 H, N-CH_2); 7.3–8.45 (m, 5 H, naphthalene). ^{13}C NMR: δ_C (250 MHz; $CDCl_3$; Me_4Si) 25.28 (1 C, -CH_2); 26.88 (1 C, -CH_2); 27.89 (1 C, -CH_2); 35.62 (1 C, -CH_2); 45.93 (1 C, -CH_3); 40.13 (1 C, -N-CH_2); 54.9 (1 C, N-CH_2-aryl); 52.7 (1 C, N-CH_2-aryl); 115,04, 116.9, 123,3, 125.6, 126.2, 129.8, 130.1, 131.05, 132.5, 155.8, 163.9, 164.4 (12 C, naphthalimide); 179.3 (1 C, COOH). MS: (FAB in nba) *m*/*z* (%): 410 [M + 1].

Synthesis of 2

For the reaction with hydrogel, **1** was dissolved in dimethylformamide at room temperature. Then 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate (for activation) and *N*-ethyldiisopropylamine (as the base) were added. Then this solution

was stirred with the hydrogel-glass-plate for 10 minutes to give **2**, which was cleaned by washing with acetone.

Synthesis of 3

4-Bromo-1,8-naphthalic acid anhydride was suspended in ethanol, the suspension was warmed to a clear solution and then a glass plate with amino-functionalised hydrogel was inserted to this solution. After refluxing for 8 hours the glass plate was washed with ethanol and acetone. Then the glass plate was heated in 2-methoxy-ethanol with an excess of 1-methyl-piperazine and after 10 hours it was washed with acetone to give substrate **3**.

Synthesis of 6-(6-morpholino-1,3-dioxo-1*H*-benzo[*de*] isoquinolin-2(3*H*)-yl) hexanoic acid 4

6-(6-Bromo-1,3-dioxo-1*H*-benzo[*de*]isoquinolin-2(3*H*)-yl) hexanoic acid, the intermediate in synthesis of **1**, was refluxed for 8 hours with an equimolar amount of morpholine in 2-methoxy-ethanol to give **4**. This product was purified on silica gel using dichloromethane/methanol 10:1 to give **4** as a yellow solid. Yield: 85%. UV absorption: $\lambda_{max}(H_2O)$/nm: 380. MS: (FAB in nba) *m*/*z* (%): 396 [M].

Spotting of 2

Equimolar amounts of **1**, 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate, hydrogel and *N*-ethyldiisopropylamine were dissolved in *N*,*N*-dimethylformamide, and after stirring 10 minutes at room temperature the mixture was spotted on glass plates and afterwards washed with ethanol.

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