Pyrylium monolayers as amino-reactive platform

Francesca A. Scaramuzzo,[a,b] Arántzazu González-Campo,[a] Chien-Ching Wu,[c,d] Aldrik Velders,[c] Vinod Subramaniam,[d] Giancarlo Doddi,[b]* Paolo Mencarelli,[b] Mario Barteri,[b]* Pascal Jonkheijm,[a]* Jurriaan Huskens [a]*

[a] *Molecular Nanofabrication Group, University of Twente, P.O. Box 217, 7500 AE, Enschede, The Netherlands. Fax:+31534894645; Tel:+31534892987; E-mail:p.jonkheijm@utwente.nl, j.huskens@utwente.nl.*
[b] *Department of Chemistry, University "La Sapienza", P.le A. Moro, 5, 00195 Rome, Italy. Fax: +396490631; Tel: +39649913957; E-mail: mario.barteri@uniroma1.it, giancarlo.doddi@uniroma1.it.*
[c] *Supramolecular Chemistry and Technology, University of Twente, P.O. Box 217, 7500 AE, Enschede, The Netherlands.*
[d] *Biophysical Engineering, University of Twente, P.O. Box 217, 7500 AE, Enschede, The Netherlands.*

Synthesis of 2,6-[4-bromophenyl]-4-[4-(undec-10-ynoyloxy)phenyl]pyrylium (compound 1):

p-hydroxybenzaldehyde (560 mg, 4.59 mmol) and *p*-bromoacetophenone (1.827 g, 9.18 mmol) in ratio 1:2 were dissolved in 7.0 mL of acetic anhydride. Seven equivalents of perchloric acid 60% (3.52 mL) were added to the mixture, which was stirred for 4 h at room temperature. The precipitate, purified by dissolving in acetone and subsequent precipitation in diethyl ether for at least 3 times, was a mixture of 2,6-[4-bromophenyl]-4-[4-acetoxyphenyl]pyrylium and 2,6-[4-bromophenyl]-4-[4-hydroxyphenyl]pyrylium perchlorates. The brown powder was totally hydrolyzed to 2,6-[4-bromophenyl]-4-[4-hydroxyphenyl]pyrylium cation in sulphuric acid 80% o.n. at room temperature. The mixture was purified by dissolving in acetone and subsequent precipitation in diethyl ether for at least 3 times. A further purification from non-phenolic impurities was performed by dissolving the solid in acetonitrile and adding 3 equivalents of triethylamine. The red/violet precipitate was dissolved in chloroform and washed 3 times with water; the organic phase was dried with sodium sulphate anhydrous, then filtered off and evaporated. The solid was then resuspended in acetonitrile and drops of fluoboric acid (50%) were added until the solution turned yellow and the compound was completely dissolved. By addition of an excess of diethyl ether the hydroxyphenyl derivative was obtained as solid.

^{1}H NMR (300 MHz, d_3-Acetonitrile, 25°C): δ = 8.55 (s, 2 H), 8.32 (d, ^3J(H,H) = 9.0 Hz, 2 H), 8.24 (dd, ^3J(H,H) = 6.8 Hz, ^4J(H,H) = 2.0 Hz, 4 H), 7.93 (d, ^3J(H,H)=6.8 Hz, ^4J(H,H)=2.0 Hz, 4 H), 7.16

ppm (d, ^{3}J(H,H) = 9.0 Hz, 2 H). ^{13}C NMR (75 MHz, d_3-Acetonitrile, 25°C), δ = 168.43, 165.46, 164.47, 133.21, 133.12, 129.90, 129.64, 128.28, 123.55, 113.58 ppm.

The final esterification was performed by stirring 2,6-[4-bromophenyl]-4-[4-hydroxyphenyl]pyrylium (150 mg, 0.263 mmol), 10-undecynoic acid (62 mg, 0.342 mmol) and dicyclohexilcarbodiimide (DCC) (543 mg, 2.63 mmol), in ratio 1:1.3:10, in a mixture of anhydrous acetonitrile/dichlorometane 1:4 o.n. at room temperature. 4-4'-Dimethylaminopyridine was added as catalyst (1.5 mg, 0.012 mmol, 0.045 equivalents). The solvent was evaporated under vacuum and the residue was dissolved in the minimum amount of acetonitrile and filtered off. Subsequent addition of diethyl ether allowed the precipitation of the solid, which was further purified through at least 3 cycles of dissolution in acetone and precipitation in diethyl ether. Yield: 31%.

^{1}H NMR (300 MHz, d_3-Acetonitrile, 25°C): δ = 8.69 (s, 2 H), 8.36 (d, ^{3}J(H,H) = 8.9 Hz, 2 H), 8.29 (d, ^{3}J(H,H) = 8.8 Hz, 4 H), 7.96 (d, ^{3}J(H,H) = 8.8 Hz, 4 H), 7.50 (d, ^{3}J(H,H) = 8.9 Hz, 2 H), 2.65 (t, ^{3}J(H,H) = 7.4 Hz, 2 H), 1.70-1.80 (m, 3 H), 1.20-1.60 (m, 12H). ^{13}C NMR (75 MHz, d_3-Acetonitrile, 25°C), δ = 171.74, 169.84, 165.39, 156.77, 133.28, 131.55, 130.22, 129.93, 127.95, 123.64, 115.87, 84.56, 68.61, 33.78, 33.42, 28.81, 28.59, 28.37, 28.25, 25.42, 24.75, 17.73 ppm. MS (ESI-ToF): m/z calculated for $C_{34}H_{31}Br_2O_3$ 647, found 647 [M^+]. Elemental Analysis (Eager 200, CE Instruments): calculated for $C_{34}H_{31}BBr_2F_4O_3$ C=55.58%, H=4.26%, found C=55.43%, H=4.29%.

Figures S1 and S2 show the UV-Vis and fluorescence spectra of compound **1**, respectively.

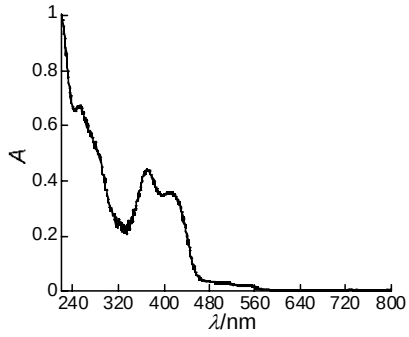

Figure S1: UV-Vis spectrum of compound **1** 1.25 x 10^{-5} M in acetonitrile.

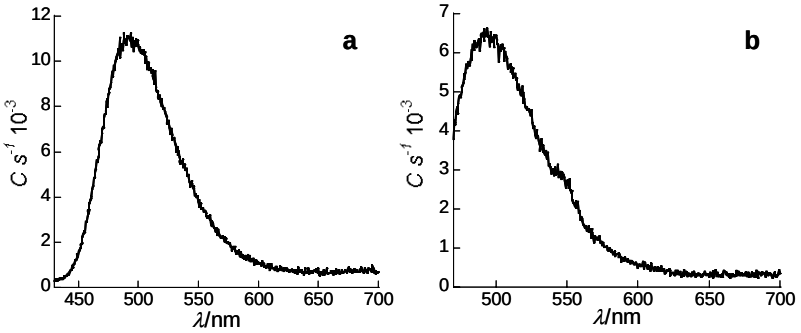

Figure S2: Fluorescence emission spectra of compound **1** 1.25 μM in acetonitrile under excitation at 410 nm (a) and 450 nm (b) respectively.

Immobilization of 1 onto an azide monolayer: XPS characterization

After printing a full monolayer of compound **1** according to the scheme reported in Figure 2, a peak at 74 eV, which is attributed to the Br 3d electrons, appears in the XP spectrum (Figure S3a). This peak remains unaltered after reaction with an aliphatic non-fluorescent amine (Figure S3b).

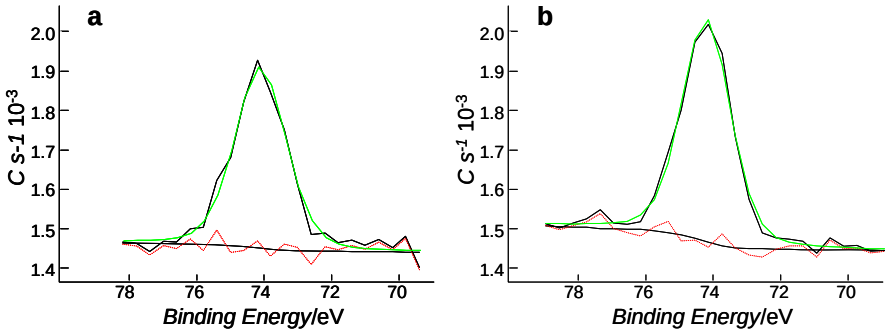

Figure S3: Br 3d XPS signal of a monolayer of **1** before (a) and after (b) the reaction with amines: measured data (black upper curves), fitting with a Gaussian-Lorentzian line shape (green curves), background line used to calculate the area under the curve (lower black lines) and misfit of the fit with the measured data (red lines).

UV-Vis characterization

UV-Vis characterization was performed over 5 quartz slides all functionalized on both sides (Figure S4). After printing on the azide layers (A) it was possible to notice a broad band between 370 and 480 nm (B), assigned to compound **1** (see Figure S1 for comparison). Reaction with LRB-EDA (5 min inking of a 70 μM solution, 10 min printing) led to the appearance of a peak clearly due to the presence of this species (C).

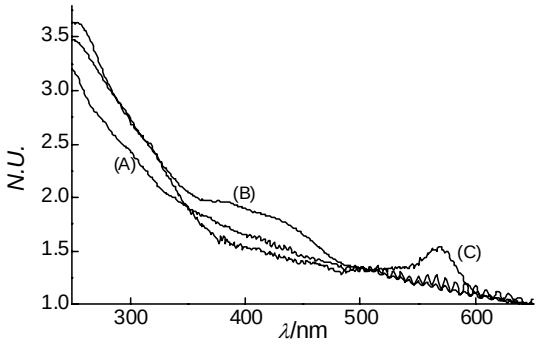

Figure S4: UV-Vis spectra of an azide layer (A), of a pyrylium layer printed on it (B) and of the pyridinium species formed after reaction with LRB-EDA (C).

Immobilization of 1 onto an azide monolayer: blank in fluorescence microscopy

To prove that the acetylenic functional group of compound **1** is involved in the reaction on the surface, 2,4,6-triphenylpyrylium chloride, lacking the acetylene moiety, was printed in lines on the azide layer using the same experimental conditions (5 min inking of a 1 mM solution, 60 min printing). As it is possible to notice from Figure S5, in this case no relevant fluorescence pattern was detected.

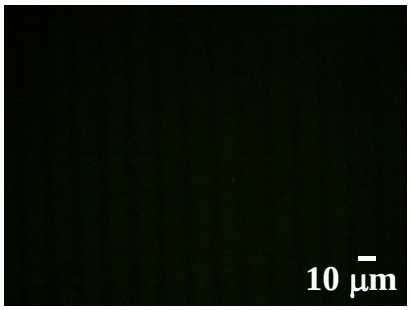

Figure S5: Fluorescence microscopy image of a 2,4,6-triphenylpyrylium chloride pattern (10 x 5 μm line features) printed over an azide layer (λ_{exc} = 460-490 nm, λ_{obs} = 520 nm, t_{exp} = 1300 ms).

Reaction with LRB-EDA: blank in fluorescence microscopy

Compound **1** does not show any fluorescence emission at λ_{obs} = 590 nm under excitation at λ_{exc} = 510-550 nm (Figure S6).

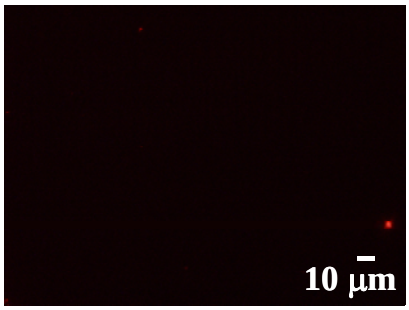

Figure S6: Fluorescence microscopy image of a compound **1** pattern (10 x 5 μm line features) at λ_{exc} = 510-550 nm, λ_{obs} = 590 nm, t_{exp} = 500 ms).

While at this wavelength printing lines of LRB-EDA over a full monolayer of compound **1** resulted in a clear pattern (Figure 3c), printing of LRB in the same experimental conditions (5 min inking of a 70 μM solution, 10 min printing) did not determine the appearance of any relevant fluorescence signal, thus demonstrating that the binding involved the amino-group of LRB-EDA (Figure S7).

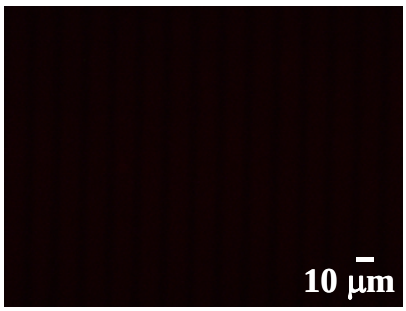

Figure S7: Fluorescence microscopy image of a LRB pattern (10 x 5 μm line features) printed over a full monolayer of compound **1** (λ_{exc} = 510-550 nm, λ_{obs} = 590 nm, t_{exp} = 200 ms).

Reaction with YFP: fluorescence intensity profile and blank

Incubation (o.n., 4°C) of a patterned monolayer of compound **1** in a solution of YFP 20 μM in PBS 10 mM led to the appearance of a fluorescence signal at λ_{exc} = 500 nm, λ_{obs} = 535 nm. The difference in the emission intensity before and after incubation with the protein is clearly shown in the plot of the intensity profiles (Figure S8). At these excitation and emission wavelengths, the pyrylium cation itself has a very weak emission (Figure S9a).

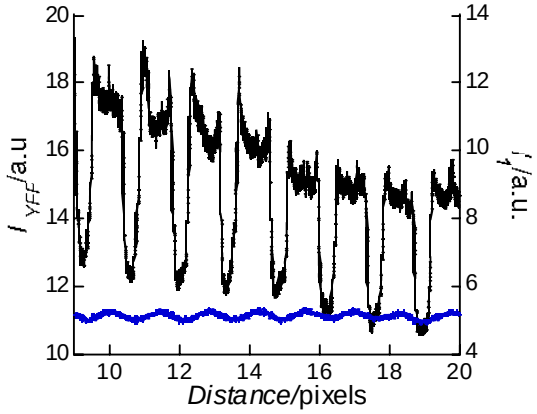

Figure S8: Fluorescence intensity profiles at λ_{exc} = 500 nm, λ_{obs} = 535 nm before (blue line) and after (black line) incubation of compound **1** with YFP.

A suitable blank, which proved that YFP is covalently bound to compound **1** through a reaction between the amino groups of the lysine residues side chains and the charged oxygen, was obtained by saturating the pyrylium rings on the surface with isopropylamine. After incubation in isopropylamine at room temperature for 3 h, compound **1** reacted completely, so that a pattern of pyridinium, much less fluorescent than pyrylium, was obtained (Figure S9b). This sample was incubated overnight at 4°C in a solution of YFP 20 μM, but no significant fluorescence of the protein has been detected (Figure S9c).

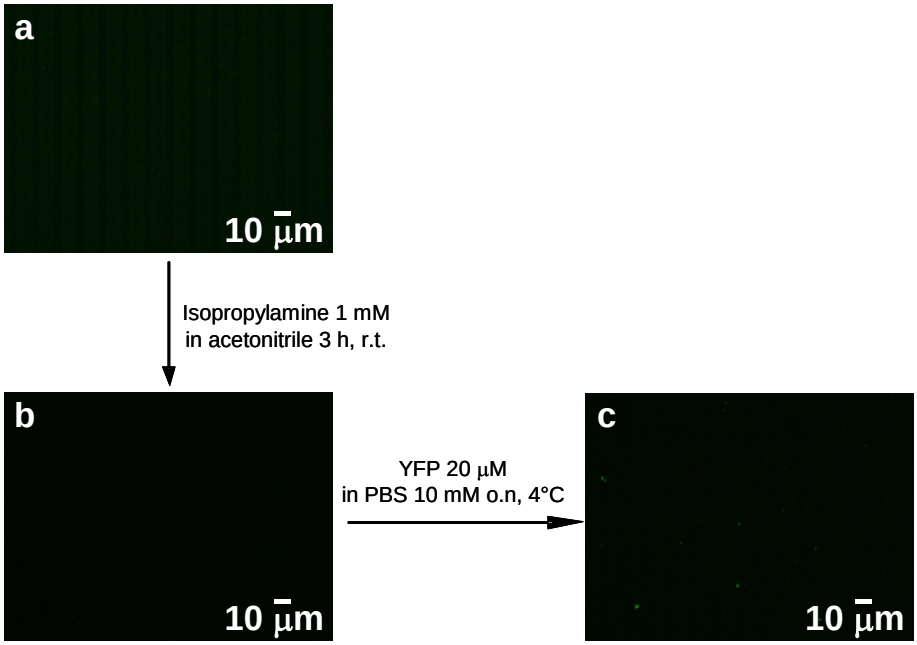

Figure S9: Fluorescence microscopy image of a pattern of compound **1** with 10 x 5 μm line features at 535 nm under excitation at 500 nm (a). After incubation in isopropylamine, the fluorescence signal was even weaker (b). In this case, YFP can not bind pyrylium, so no fluorescence emission was observed (c). For all the images, t_{exp} = 1300 ms.

DPN: comparison between LRB-EDA and LRB (blank)

DPN was used to write the same pattern with LRB-EDA and LRB over a full monolayer of compound **1** on the same conditions of temperature and relative humidity percentage (Temperature 20-23°C, relative humidity 60%). The patterns obtained were rinsed first with 2 aliquots of 80 µl each of acetonitrile and then with several aliquots of 80 µl each of ethanol. After 6 rinsing cycles the pattern written with LRB disappeared (i.e. the fluorescence signal was not different from the layer underneath), while after 10 rinsing cycles it was still possible to see the pattern written with LRB-EDA (Figure S10).

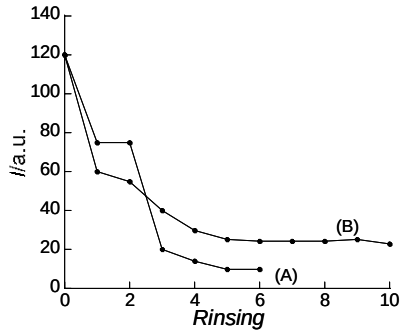

Figure S10: Fluorescence intensity of LRB (A) and LRB-EDA (B) written on a full monolayer of compound **1** after rinsing.

Hydrolysis of pyrylium monolayers.

When the pyrylium cation reacts with OH^-, the so-called pseudobase is formed which can open to yield the corresponding diketonic form, which is neutral (Figure S11). The diketone was obtained by adding 1 equivalent of aqueous NaOH to a 1 mM solution of pyrylium in acetonitrile. We performed the experiment in 1 mM NaOH or 1 mM HCl. After immersion for 1 h 40 min, the fluorescence was constant, indicating that the reaction was complete. A scheme of the experiment is shown in Figure S12.

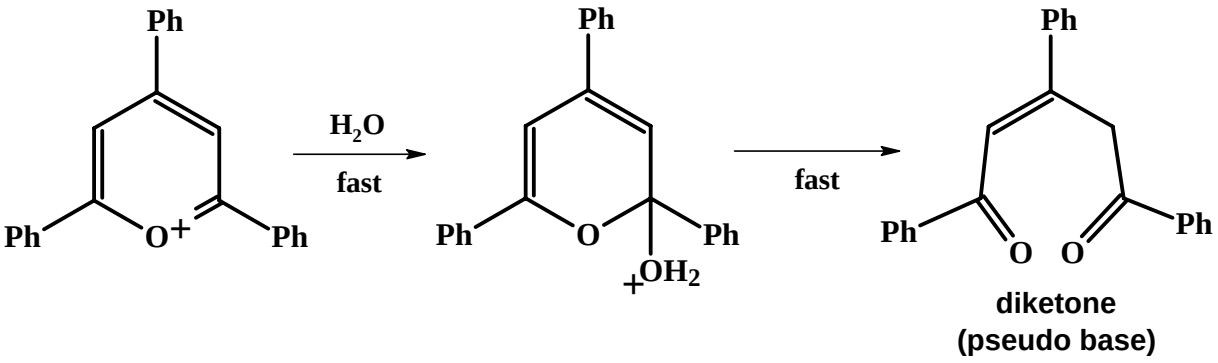

Figure S11: Scheme of the pseudobase and of the corresponding diketone formation.

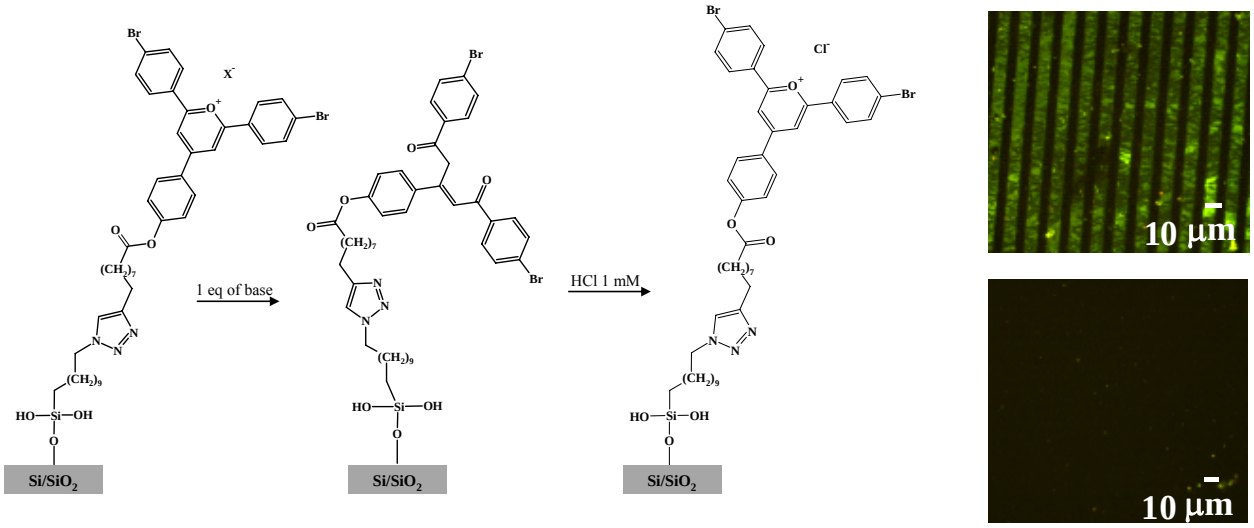

Figure S12: Scheme of ringopening and closure of pyrylium monolayers. Fluorescence images of pyrylium patterns before and after (top) ring closure and of diketone monolayer (bottom).

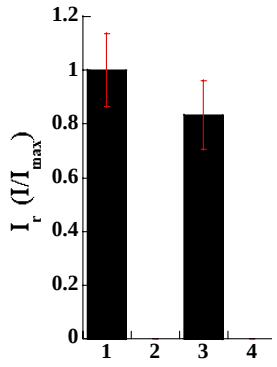

Figure S13: Normalized fluorescence intensity vs number of ring opening/closure steps. In this case the alkaline medium used is NaOH. Odd number steps refer to the ring closure step, while even number steps refer to the ring opening step. The results were analyzed in the following way: for each sample, the average fluorescence intensity <I> was expressed as an average value over 5 lines of 14 μm (2800 points each). The error at this stage was considered to be the standard deviation. For each condition (HCl/NaOH or HCl/Et_3N treatment) we averaged the values obtained from two samples. Every time the blank, i.e. the <I> value in the non-printed zones, was subtracted. As no pattern could be detected in the ring opening step, the fluorescence intensity can be considered zero after blank subtraction. In the end, the values obtained were normalized for the maximum value and the final error was expressed as a relative error. No meaningful differences between samples treated with NaOH or Et_3N are evident, therefore only the results for the HCl (odd cycle numbers) /NaOH (even cycle numbers) treatment are reported.

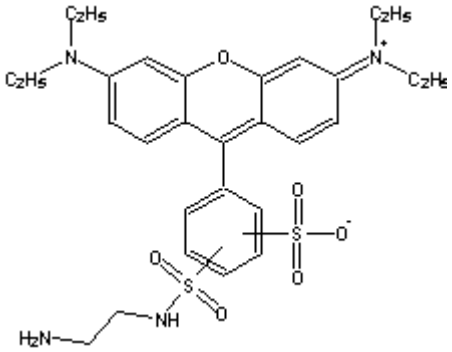

Figure S14. Molecular structure LRB-EDA.