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Post-print of a peer-reviewed article published by the Royal Society of Chemistry.
Published article available at: http://dx.doi.org/10.1039/B924151E

Cite as:
Graham, E. M., Iwai, K., Uchiyama, S., de Silva, A. P., Magennis, S. W., & Jones, A. C. (2010). Quantitative mapping of aqueous microfluidic temperature with sub-degree resolution using fluorescence lifetime imaging microscopy. *Lab on a Chip*, 10(10), 1267-1273.

Manuscript received: 17/11/2009; Accepted: 03/02/2010; Article published: 25/02/2010

Quantitative Mapping of Aqueous Microfluidic Temperature with Sub-degree Resolution Using Fluorescence Lifetime Imaging Microscopy**

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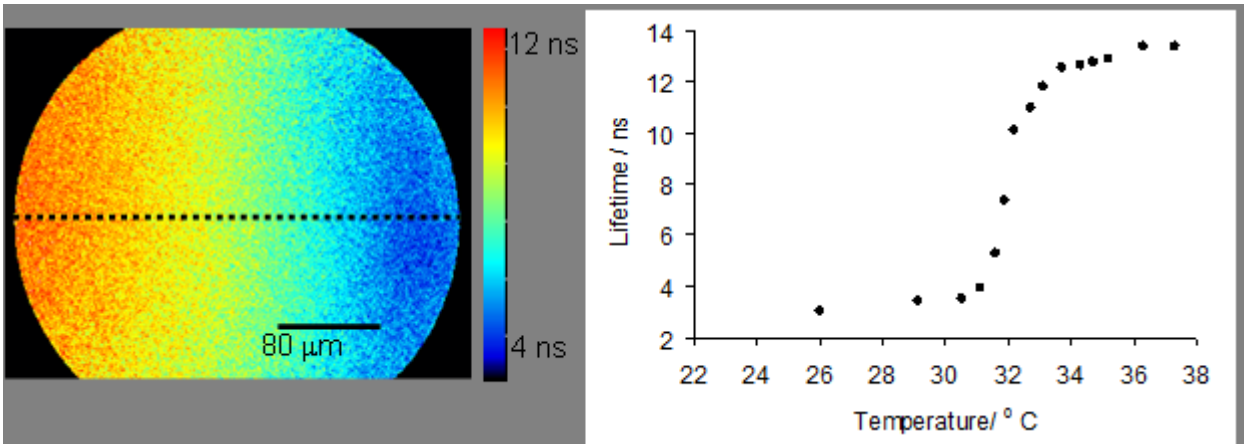
[**]This work was supported by the EPSRC, Insight Faraday Partnership, SFC, Lab 901 Ltd, Japan Society for the Promotion of Science and InvestNI (RTD COE 40). We would like to thank Mr. Andy Garrie for fabricating the flow cell. SWM acknowledges the award of an EPSRC advanced research fellowship (EP/D073154).

Supporting information:
†Electronic supplementary information (ESI) available: Fig. S1, Tables S1 and S2 at

http://dx.doi.org/10.1039/B924151E

Graphical abstract



Synopsis

The temperature of aqueous solutions in microfluidic devices can be mapped, on the micron scale, with a resolution of less than 0.1 °C by using fluorescence lifetime imaging microscopy (FLIM), and a water-soluble, thermo-responsive polymer as a lifetime probe.

Abstract

The use of a water-soluble, thermo-responsive polymer as a highly sensitive fluorescence-lifetime probe of microfluidic temperature is demonstrated. The fluorescence lifetime of poly(N-isopropylacrylamide) labelled with a benzofurazan fluorophore is shown to have a steep dependence on temperature around the polymer phase transition and the photophysical origin of this response is established. The use of this unusual fluorescent probe in conjunction with fluorescence lifetime imaging microscopy (FLIM) enables the spatial variation of temperature in a microfluidic device to be mapped, on the micron scale, with a resolution of less than 0.1 °C. This represents an increase in temperature resolution of an order of magnitude over that achieved previously by FLIM of temperature-sensitive dyes.

Introduction

The development and use of microfluidic reactors in biological and chemical research has dramatically increased in recent years because of the benefits of performing reactions at the microscale.[1,2] The main advantage of miniaturising temperature-dependent reactions is a consequence of the high surface-area-to-volume ratio which allows rapid heating and cooling of reaction chambers under isothermal conditions with well-defined residence times.[3] Countless biological processes and chemical reactions require precise temperature control, to guarantee successful yields and to ensure consistent and repeatable performance.[4]

Microfluidic devices have proved very successful in handling and manipulating individual biological cells in solution.[5,6] The control of temperature within such devices is critical, as temperature increases of only a few degrees above the physiological temperature can cause the production of cellular heat shock proteins and cell damage.[7] In the past decade, numerous lab-on-a-chip devices have been developed to perform polymerase chain reaction (PCR) which is used for the amplification of DNA in molecular biology, chemical and biomedical analysis, gene diagnostics and genetic identification.[8-11] DNA amplification by PCR involves thermal cycling through three temperatures phases: denaturation, annealing and DNA synthesis by a thermostable polymerase. The control of temperature is essential in PCR. For example, if the annealing temperature is slightly too high, the purity and yield of product can be reduced, whereas too low a temperature can cause amplification of non-specific DNA fragments. These effects have been shown with deviations of as little as 1 ^{o}C from the optimum annealing temperature.[9]

While thermal management is clearly an extremely important consideration for the design, fabrication and performance of microfluidic devices, it can be problematic to accurately measure temperature in the extremely small volumes of fluids within these devices.[12] The most common methods rely on external or integrated micro-thermocouples.[13,14] Surface-mounted sensors have been found generally to be ineffective at providing reliable temperature information, as the surface temperature of a device can differ greatly from the internal temperature of the fluid.[2,4,15-19] Thermocouples inserted into devices can perturb the fluid temperature, because of the large thermal capacity of the thermocouple compared with that of the channel or chamber, resulting in large errors in the fluid temperature measurement,[20] and can also inhibit certain types of biochemical reactions.[17] This approach is also limited to measuring the temperature at only a few discrete points in the microfluidic device.[10,17,19]

Optical interferometry, exploiting the temperature dependence of the refractive index of water,[21] imaging of thermochromic liquid crystal suspensions,[2,10,12,19] optical detection of the phase transition of polymer solutions[7,22] and a number of spectroscopic methods, including nuclear magnetic resonance (NMR),[23,24] Raman[20,25,26] and fluorescence, have been used for the non-invasive measurement of temperature. Amongst spectroscopic methods, fluorescence offers the highest detection sensitivity, enabling high spatial resolution and short acquisition times, making it an attractive technique for monitoring microfluidic conditions.

Fluorescence methods are generally based on the temperature-dependence of fluorescence intensity and have included the use of DNA molecular beacons[15,17,27,28] and a surface coating of a ruthenium complex.[29,30] A popular method for both temperature imaging and single-point measurements exploits the temperature-dependence of the fluorescence quantum efficiency of rhodamine B (RhB) solutions. The free rotation of the diethylamino group in the xanthene chromophore of RhB facilitates internal conversion from the first excited state to the ground state, the main non-radiative deactivation pathway, leading to temperature dependence of the fluorescence lifetime and quantum yield.[31] There have been numerous publications reporting the use of the fluorescence intensity of RhB to measure temperature within microfluidic devices.[7,16,18,32-34] However, the

ability of fluorescence intensity-based techniques to perform reliable quantitative measurement of microfluidic temperature is severely compromised by their sensitivity to variations in the optical path, instability of the light source, scattering, uncertainty in the dye concentration, and photobleaching effects. The measurement of fluorescence lifetime, rather than intensity, overcomes these problems and fluorescence lifetime imaging microscopy (FLIM) is becoming established as a powerful method for the quantitative examination of microfluidic properties.[35-44] Benninger *et al.* have reported the use of FLIM to image the temperature of RhB in methanolic solution within a glass microchip,[38] designed to perform continuous-flow PCR reactions. Quantitative profiles of temperature with respect to both width and depth of microchannels were obtained with a precision of ±1 ^{o}C. The low solubility of RhB in water makes it unsatisfactory for measuring aqueous microfluidic temperature because of problems with deposition on surfaces.[12] However, Mendels *et al.* have used Kiton Red, a water-soluble derivative of RhB that shows the same lifetime-temperature response as the parent fluorophore, for FLIM of aqueous microfluidic systems.[44] They used quantitative FLIM measurements of temperature and fluid composition in combination with computational fluid dynamics to study thermal and solutal transport processes in a microfluidic T-mixer. RhB and Kiton Red fluorophores are useful and effective fluorescence lifetime probes of temperature over the range 10 to 100 ^{o}C; however, the relatively weak dependence of their fluorescence decay on temperature, which obeys the Arrhenius equation,[44] limits the temperature resolution to 1-2 ^{o}C. In this paper, we report the use of an unusual fluorescent polymer probe to resolve temperature changes of aqueous solutions in microfluidic devices that are an order of magnitude smaller than can be measured with RhB.

Previously it was shown that certain N-alkylacrylamide polymers labelled with a benzofurazan fluorophore show an extremely sensitive dependence of fluorescence intensity on temperature and can be used as responsive and reproducible fluorescent molecular thermometers.[45-47] More recently, a hydrophilic nanogel variant of one of these polymers was used as an intracellular thermometer, which could resolve temperature differences of less than 0.5 °C in living cells.[48] The N-alkylacrylamide polymers in aqueous solution undergo a phase transition above a lower critical solution temperature (LCST), causing a considerable decrease in microenvironmental polarity in the vicinity of the main polymer chain.[49] The fluorescence properties of benzofurazan are affected by solvent polarity; its fluorescence intensity increases sharply with decreasing polarity.[50] Thus, copolymerising a small amount of a benzofurazan monomer with an N-alkylacrylamide results in a fluorescent copolymer that shows a dramatic increase in fluorescence intensity with increasing temperature over a range of about 10 ^{o}C around the LCST. Although the operational temperature range of this type of molecular thermometer is narrow, it has been shown that by using N-alkylacrylamides with different LCSTs and co-polymers of two different N-alkylacrylamides, the temperature range between 18 and 54 °C can be covered.[46]

In this study, we investigate the copolymer of 4-N-(2-acryloyloxyethyl)-N-methylamino-7-N,N-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-AE), shown in Fig. 1a, with N-isopropylacrylamide (NIPAM) as a fluorescence lifetime probe of temperature in microfluidic devices. Of several different

benzofurazan monomers investigated, DBD-AE showed the most sensitive fluorescence response to the poly(N-alkylacrylamide) phase transition.[45] Poly(NIPAM), shown in Fig.1b, undergoes a phase transition in aqueous solution with a LCST of ~32 °C, and the copolymer with DBD-AE, poly(DBD-AE-*co*-NIPAM), has been shown to exhibit 13-fold increases in fluorescence intensity between 29 and 37°C.[45] Although intensity change often predicates a change in lifetime, the two do not necessarily go together. For example, interconversion between fluorescent and non-fluorescent forms affects intensity but not lifetime. A recent study of a related fluorescent polymer, poly(DBD-AA-*co*-NIPAM), where DBD-AA is N-{2-[(7-N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazol-4-yl](methyl)amino}ethyl-N-methylacrylamide, demonstrated that the fluorescence lifetime was extremely sensitive to temperature, and established that the increase in lifetime with increasing temperature was due to both decrease of local polarity around the DBD-AA unit and the loss of hydrogen bonding between DBD-AA and water.[51] In this report, we investigate the time-resolved fluorescence of poly(DBD-AE-*co*-NIPAM), providing insight into the photophysical response of the polymer to temperature and establishing its potential as a FLIM temperature probe. We then demonstrate its use as a FLIM probe to map microfluidic temperature with a resolution of less than 0.1 ^{o}C, which makes it the most sensitive fluorescent molecular thermometer to date.

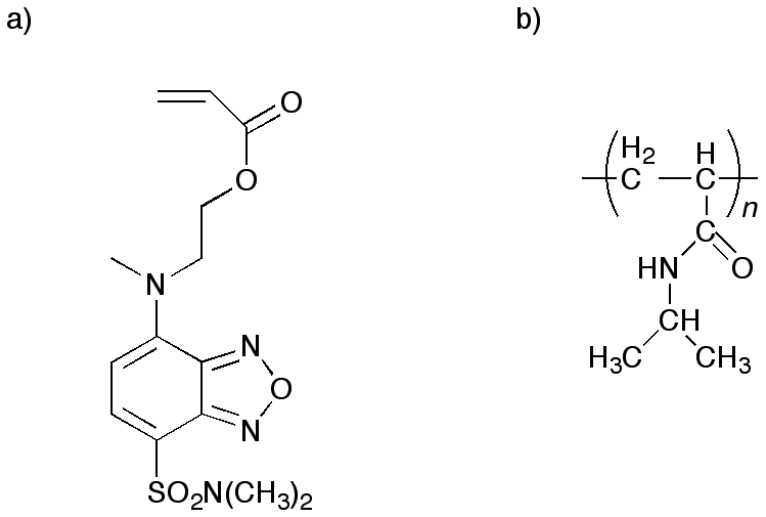


Figure 1. Chemical structures of (a) DBD-AE and (b) poly(NIPAM)

Experimental

Materials

The copolymer, poly(DBD-AE-*co*-NIPAM) was prepared as previously described.[45] It was used as an aqueous solution of concentration 0.2% w/v. The solution was prepared using HPLC grade water (Fisher

Scientific, used as received), and stored in the dark. The fluorescence lifetime was used as a routine check of sample purity after storage. No fluorescence emission, at the wavelengths employed, could be detected from the solvent under the instrumental conditions used.

Microfluidic System

A moulded polymer-based micro fluidic chamber (provided by Lab 901 Ltd) was filled with poly(DBD-AE-co-NIPAM) solution. The solution was injected (from a 1 ml luer syringe fitted with a 0.4 mm diameter needle, both from BD) through an inlet channel to fill the chamber. The inlet and outlet channels were then heat-sealed. A temperature gradient was achieved by applying parallel heating and cooling elements to either side of the chamber. The left-hand side of the chamber was heated by contact with a nichrome wire element (0.71 mm diameter). The temperature of the wire was controlled by varying the voltage supplied by a DC power supply (Thander TS3022). A cold sink was achieved at the right-hand side of the chamber through contact with a copper rod, cooled with ice packs. The cold sink and parallel heat source created a linear temperature gradient across the chamber.[29]

Fluorescence spectroscopy and microscopy

The excitation source was a Ti-Sapphire femtosecond laser system from Coherent (10 W Verdi and Mira Ti-Sapphire laser) producing pulses of ca. 200 fs at 76 MHz. The output of the Mira was passed through a pulse picker (reducing the repetition rate to 4.75 MHz) and then frequency doubled to give an output at 430 nm. The excitation beam was split, and one portion was used to trigger a fast photodiode.

FLIM

For FLIM the excitation light was expanded, collimated and directed into an inverted microscope (Nikon TE300) operating in an epifluorescence configuration. This excitation light was reflected from a dichroic filter (DM455, Nikon) and focused into the microfluidic device using either a 4x (PA, NA=0.2), 10x (PA, NA=0.45), 20x (PA, NA=0.75), 40x (PF, NA=0.60) or 50x (LU Plan, NA=0.80) Nikon objective. The resultant fluorescence was collected through the same objective, passed through a barrier filter (590 nm, Nikon) and imaged onto a Picostar HR-12QE gated intensified CCD camera system (LaVision GmbH, Berlin) using a setup that has been described previously.[35] The FLIM images were recorded with a 600 ps gate width (which was measured by detecting laser light reflected from a mirror). Images were the average of 5 separate exposures employing 4 x 4 binning and were recorded in steps of 1 ns over a 51 ns range. The camera exposure time for each image was 100 ms. The excitation intensity was adjusted to give a peak intensity of

between 3000 and 4000 counts in the brightest image, which corresponds with the start of the fluorescence decay. The background signal of *ca.* 50 counts was subtracted from each image. The Picostar system and delay card were controlled, and the data was analysed, using DaVis 6.2 software running the Picostar DaVis module.

The instrument response function (IRF) was recorded, by using a mirror in place of the sample, for each of the gate widths used. It was thus established that the fluorescence decay could be fitted from 1 ns after the emission peak image without distortion by the instrument response. The images were analysed by constructing a decay curve for each pixel. Pixels with low counts in the first analysed image were removed by adjusting the threshold. A lifetime map was produced by assigning a colour on a 16-bit pseudocolour scale to the fitted single exponential decay times.

Time-resolved fluorescence spectroscopy

Fluorescence lifetimes of bulk solutions were measured using the technique of time correlated single photon counting (TCSPC), in an Edinburgh Instruments spectrometer equipped with TCC900 photon counting electronics. The sample solution was contained in a 1 cm path-length fused silica micro-cuvette (10 x 2 mm inside dimensions). The temperature of the solution was controlled by circulating water, from a thermostatically controlled water bath (Grant LTD6), through the cuvette holder. The solution temperature was measured with a thermocouple (Hanna Instruments HI93530) placed directly in the sample cuvette. The temperature of the solution was monitored until a constant value was attained.

Fluorescence was excited at 430 nm and detected at 580 nm. The emission from the sample was collected at right angles to the excitation direction, through a polariser set at the magic angle with respect to the vertical polarisation of the incident beam. The fluorescence was passed through a monochromator (bandpass 10 nm) then detected by a Hamamatsu MCPPMT (R3809U-50). A long-pass filter (Schott) was placed between the sample and the monochromator to eliminate laser light scattered by the polymer molecules.

The instrument response of the system, measured using a Ludox scatterer, was approximately 70 ps FWHM. Fluorescence decay curves were recorded over a 100 ns time range, resolved into 4096 channels, and were accumulated to give 10,000 counts in the maximum channel. The time-resolved data were fitted to a multiexponential decay function (Eq. 1):

$$I(t) = \sum_{i=1}^{n} A_i \exp\left(\frac{-t}{\tau_i}\right) + B \qquad (1)$$

where τ_i is the fluorescence lifetime and A_i (the "A-factor") is the fractional amplitude of the i^{th} decay component, and B is the background (dark count of the detector). The A-factor indicates the fraction of the

emitting molecules that has a particular lifetime, τ_i. Analysis of individual decay curves was performed with F900 software (Edinburgh Instruments), whilst global analyses were performed using FAST software (Edinburgh Instruments). In global analysis, a set of decay curves is fitted simultaneously, with lifetimes, τ_i, as common parameters. The quality of the fits was determined by the value of the reduced chi-squared statistical parameter and by visual inspection of residuals.

Results and discussion

Time-resolved fluorescence spectroscopy of poly(DBD-AE-*co*-NIPAM): The fluorescence decay of the benzofurazan fluorophore within the poly(DBD-AE-*co*-NIPAM) was measured as a function of temperature over the range 22.9 to 38.3 ^{o}C. It was found that the decay was multiexponential at all temperatures, requiring three lifetime components to give a satisfactory fit. The overall dependence of the fluorescence decay time on temperature can be represented by the average lifetime, $\Sigma A_i\tau_i/\Sigma A_i$, which increases sharply in the region of the LCST, as shown in Fig. 2. The average lifetime increases from 3.0 ns at 22.9 °C to 13.5 ns at 38.3 °C. In the steepest section of the phase transition there is a change in average lifetime of 1 ns for 0.1 ^{o}C increase in temperature. There is a quasi-sigmoidal relationship between lifetime and temperature, which is similar to the temperature-intensity relationship.[45] The strong dependence of the average lifetime on temperature enables poly(DBD-AE-*co*-NIPAM) to be used in conjunction with FLIM as a sensitive probe of temperature.

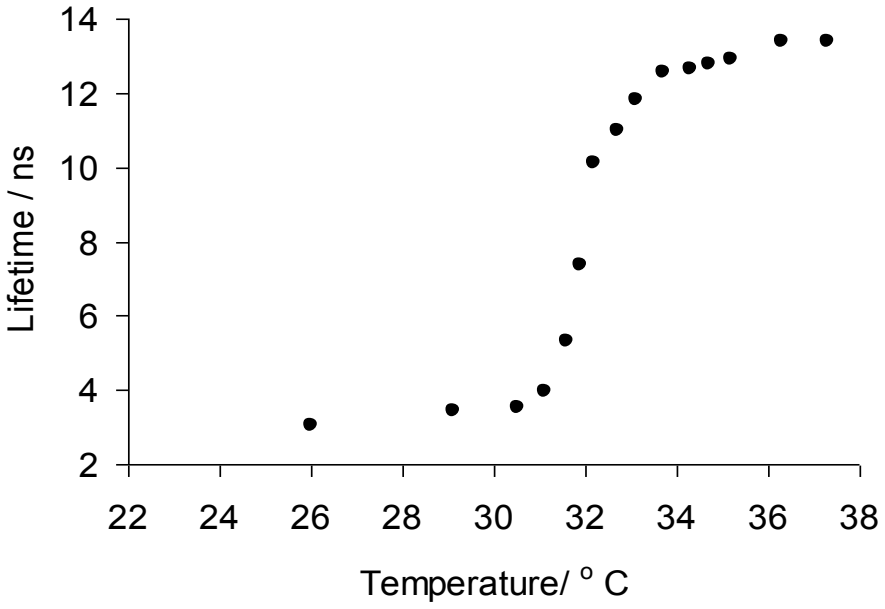


Figure 2. Dependence of the average fluorescence lifetime of poly(DBD-AE-*co*-NIPAM) in aqueous solution on temperature

Global analysis of the decay curves showed that they could all be fitted well by the same three lifetimes, 2.16, 6.02 and 15.1 ns, with the fractional amplitude (A factor) of each component varying with temperature. This shows that the same three emitting species coexist over the entire temperature range and that the effect of temperature is to change their relative populations. The results of global analysis are presented graphically in Fig. 3, which shows the temperature dependence of each A factor.

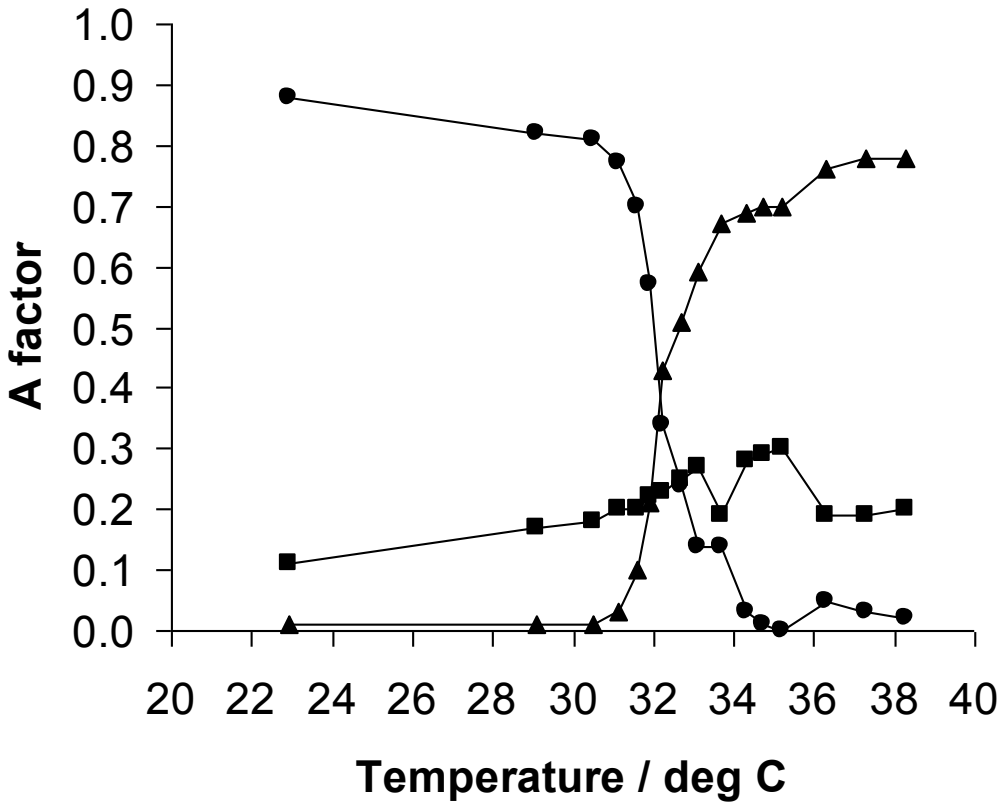


Figure 3. Temperature dependence of the amplitude (A factor) of each decay component of poly(DBD-AE-co-NIPAM) as determined by global analysis. ● 2-ns component; ■ 6-ns component; ▲ 15-ns component.

The fractional amplitude of the 6-ns component is relatively constant with temperature and represents only a small proportion of the emitting population (10-20%). In contrast, it is clear from Fig. 3 that the fractional amplitudes of the other two components depend considerably upon the temperature of the aqueous solution. At low temperature, before the phase transition has occurred, the short lifetime (2 ns) predominates (80-90%), and the long component (15 ns) is negligible (< 1%). As the temperature is increased, the contribution of the 2-ns component to the emitting population decreases, whilst that of the long lifetime species increases. At the LCST of 32 °C, the contribution of these two components is almost equal, whilst above this temperature, the

15 ns component predominates (~ 80%), and the 2-ns species is now reduced to only 1% of the total population. It is the temperature-induced change in the amplitudes of the 2-ns and 15-ns components that results in the change in average lifetime shown in Fig. 2 and the change in intensity observed in steady-state measurements.

The observation of three decay components indicates the existence of three photophysically distinct emitting species, corresponding to the benzofurazan fluorophore in different microenvironments. In reality, it is likely that each decay time represents a distribution of benzofurazan fluorophores in a range of similar microenvironments, rather than a single emitting species. The ability to fit the decay curves with 3 discrete lifetimes signifies the presence of 3 distinctly different types of microenvironment, such that the difference in decay times between these environments is great compared with the range of lifetimes within each microenvironment. The temperature dependence of the amplitudes of the 2-ns and 15-ns components can be interpreted in terms of the existence of the benzofurazan fluorophore in two different microenvironments in the vicinity of the main chain of the polymer. The fluorescence intensity is known to increase with decreasing polarity, thus the 2-ns lifetime can be attributed to benzofurazan in a polar environment, exposed to the aqueous solvent, while the 15-ns lifetime is characteristic of benzofurazan in a non-polar, hydrophobic environment, shielded from the solvent.

The phase transition results in the transfer of the fluorophore from a polar to a non-polar environment, with the evolution of the two populations reported by the temperature dependence of the respective A factors. This behaviour is consistent with previous evidence that the poly(NIPAM) chain adopts an extended coil conformation below the LCST and collapses into compact globules above the LCST.[49] The third decay component, which shows an intermediate lifetime (6 ns) and a temperature-independent amplitude implies the existence of benzofurazan in a region of the polymer with moderate polarity that is largely unaffected by the phase transition

The fluorescence decay characteristics observed here are in good agreement with a recent time-resolved fluorescence study of the related polymer, poly(DBD-AA-*co*-NIPAM), in aqueous solution;[51] multiexponential decay kinetics were reported, with an average fluorescence lifetime increasing from 3.92 ns to 14.1 ns over the temperature range 25-40 ^{o}C. The TCSPC decay data for that work were fitted with biexponential decay functions, and, in the absence of global analysis, both the fitted lifetimes and the respective A factors varied with temperature. This is in contrast to the triexponential fits required here for the poly(DBD-AE-*co*-NIPAM), which are demonstrated by global analysis to show variation in only the A factors with temperature. The similarity in the change of average lifetime with temperature in these two independent studies, suggests that the same fluorescence enhancement mechanism is, in fact, operating in both cases, and the temperature-dependence of the lifetimes of the different emitting species reported for poly(DBD-AA-*co*-NIPAM)[51] may be due to the correlation between A factors and lifetimes that occurs when multi-exponential decays are fitted individually rather than globally (see Tables S1 and S2 of the supplementary information). It

should also be noted that polarity may not be the only factor in determining the lifetime of the benofurazan fluorophore in the different microenvironments; studies of model fluorophores revealed that hydrogen bonding interactions could also be influential in these polymers.[51]

Fluorescence Lifetime Imaging (FLIM) of poly(DBD-AE-*co*-NIPAM): We now demonstrate that the extremely sensitive temperature dependence of the average fluorescence lifetime of aqueous solutions of poly(DBD-AE-*co*-NIPAM), within the phase transition range of 30 to 34 ^{o}C, can be exploited to map spatially temperature changes of a fraction of a degree within microfluidic devices. Fig. 4 shows a calibration curve of temperature versus lifetime obtained by fitting a third order polynomial function to the data presented in Fig. 2.

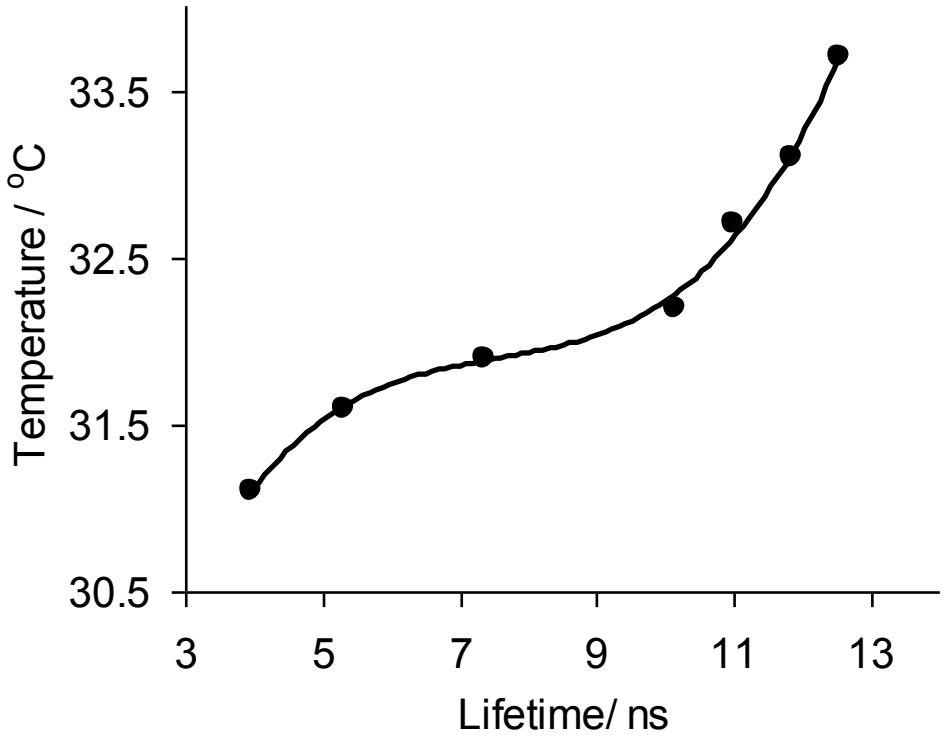


Figure 4. The calibration curve (third order polynomial) relating aqueous temperature to the average lifetime of poly(DBD-AE-*co*-NIPAM) over the range of the phase transition.

Fig. 5(a) shows the FLIM image of the entire microfluidic chamber obtained using a 4x objective. The applied temperature gradient decreases across the chamber from left to right. The FLIM image clearly shows the temperature gradient across the chamber. In the relatively broad red and blue areas, the temperature extends

into the ranges above and below the phase transition, respectively, where the lifetime response becomes flat. At the right hand side of the chamber, where cold sink is positioned, the temperature appears to increase at the wall, as shown by the yellow area at the edge of the image which indicates an increase in fluorescence lifetime. This perturbation of the FLIM image can be attributed to the detection of fluorescence that is wave-guided within the chamber wall. The wave-guided fluorescence will originate mainly from the high intensity region on the heated side of the chamber and will therefore show an average lifetime close to the upper limit, giving the false impression of elevated temperature. In Fig. 5(b), the region in the centre of the chamber where the lifetime shows a steep gradient is shown at higher magnification.

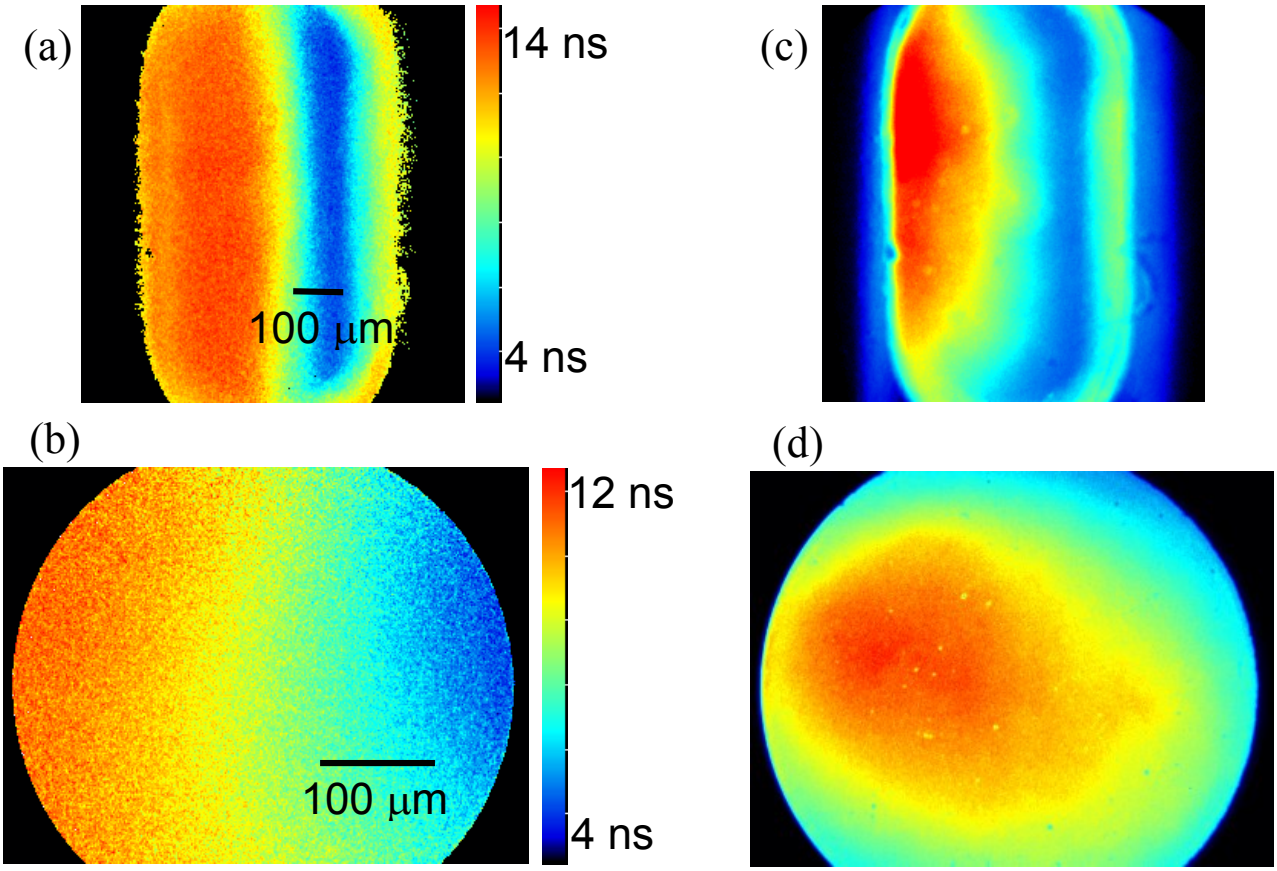


Figure 5. (a) A FLIM image showing the entire microfluidic chamber, recorded using a 4x magnification objective with an acquisition time of 127 s; (b) a FLIM image of the central region of the chamber, recorded using a 40x magnification objective with an acquisition time of 43 s; (c) the intensity image corresponding to (a); (d) the intensity image corresponding to (b). In (c) and (d) highest intensity is shown as red and lowest intensity as blue.

For comparison, the intensity image corresponding to each FLIM image has been constructed by summing all the time-gated images. These are shown in Fig. 5 (c) and (d). In principle, the intensity gradients should resemble the lifetime gradients, since the intensity and lifetime show a similar temperature dependence. However the intensity images are severely distorted because of the curvature of the chamber and the poor optical quality of the polymer from which it is fabricated. The intensity image of the entire chamber (Fig. 5(c)) gives some indication of the temperature gradient, but in the higher magnification image (Fig. 5(d)) the gradient cannot be discerned.

The FLIM images are the result of fitting the fluorescence decay at each pixel to a single exponential function and hence only give an approximate indication of temperature. To obtain a quantitative measure of temperature, decay data from a region of interest were extracted, subjected to bi-exponential fitting, the average lifetime calculated and converted to temperature using the calibration function shown in Fig. 4. The quality of the decay data from the FLIM measurements is significantly lower than that obtained by TCSPC in the cuvette-based calibration measurements, and does not permit the resolution of three lifetime components. Although three components are required to give a good global fit to the TCSPC data, an adequate fit to individual decay curves could, in fact, be obtained with two components, with negligible effect on the value of the average lifetime extracted (see Fig. S1 of the supplementary information). For example, the average lifetimes calculated from fitting the 31.9 ^{o}C decay to two and three exponentials are 7.64 ns and 7.36 ns, respectively, while the reduced chi-squared values are 1.290 and 1.078, respectively. This difference in average lifetime translates into a difference in reported temperature of only 0.02 ^{o}C. A temperature profile constructed in this fashion is shown in Fig. 6. Each point in the profile was obtained by averaging decay data from 10 (vertical) x 4 (horizontal) pixels, corresponding to an area of approximately 10 μm x 4 μm, yielding about 80 measurements along the cross-section. It can be seen that the temperature can be determined with a resolution of less than 0.1 ^{o}C in a detection volume of a few hundred femtolitres. We estimate that the precision of the average lifetime extracted from the FLIM image is 0.2ns, which corresponds to an anticipated temperature resolution ranging from 0.02 ^{o}C at the lower end of the temperature range shown in Fig. 6(b) to 0.06 ^{o}C at the higher end of the range. This is consistent with the degree of scatter apparent in the data presented in Fig. 6(b).

There is scope for achieving even higher temperature resolution by resolving smaller changes in fluorescence lifetime. The present time-gated FLIM technique is ultimately limited by the gate width of the image intensifier (*ca.* 200 ps), the restricted dynamic range of intensity that can be recorded by the CCD, the inherent difficulty in analysing the multi-exponential decays measured by such detectors, and the requirement for sequential image collection. These limitations necessitate a compromise between the resolution and range of the lifetime measurement. However, use of FLIM methodology based on time-correlated single photon counting would permit high time resolution over a wide dynamic range of lifetime, delivering higher temperature resolution without compromising temperature range.

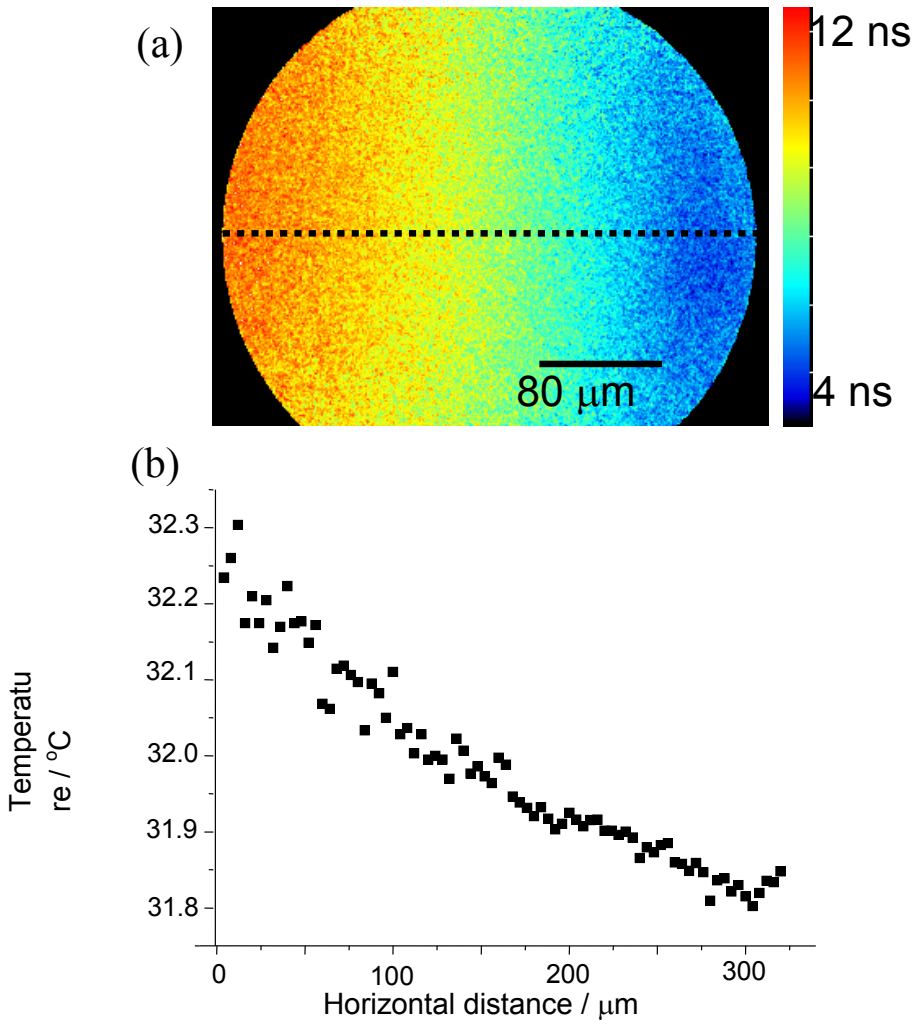


Figure 6. (a) FLIM image of the central region of the chamber recorded using a x 50 objective. The temperature profile along the cross section indicated is shown in (b).

To our knowledge, the only comparable, previous measurement of microfluidic temperature with sub-degree resolution used reflectance spectroscopy of thermochromic liquid crystals to give a resolution of 0.4 °C over a range of 5 °C.(2) Single point measurements using a fibre optic spectrometer were used to determine the temperature profile across a microreactor with a relatively low distance resolution of 0.5 mm. A similar approach was adopted subsequently to measure whole-volume temperature dynamics in 3mm-diameter chambers on a PCR chip, with a reported resolution of ~1 °C over a 3 °C range.[52] At present, therefore, FLIM of poly(DBD-AE-*co*-NIPAM) appears to be unique in combining sub-degree temperature resolution with micron-scale spatial resolution.

Conclusions

Poly(DBD-AE-*co*-NIPAM) in aqueous solution exhibits a multi-exponential fluorescence decay, the average lifetime of which is a highly sensitive probe of temperature, increasing from 3.0 ns to 13.5 ns between 23 ^{o}C and 38.3 °C. The strong temperature-dependence of the average lifetime arises from the change in the microenvironment of the benzofurazan fluorescent label as a consequence of the polymer phase transition. The phase transition results in transfer of the fluorophore from an aqueous environment, where it has a fluorescence lifetime of ~2ns, to a hydrophobic environment, where it has a lifetime of ~15ns. The temperature-dependent average lifetime thus reflects the change in the amplitudes of the 2-ns and 15-ns decay components across the phase transition.

The use of poly(DBD-AE-*co*-NIPAM) in combination with FLIM enables the spatial variation of microfluidic temperature to be mapped on the micron-scale with a temperature resolution of <0.1 ^{o}C. The water solubility of poly(DBD-AE-*co*-NIPAM) and related benzofurazan-labelled poly(N-alkylacrylamide)s and their response at physiologically-relevant temperatures will make them particularly valuable as FLIM probes for use in the development of lab-on-a-chip devices for biomedical applications in which precise temperature control is crucial.

References

[1] A. J. De Mello, M. Habgood, N. L. Lancaster, T. Welton and R. C. R. Wootton, *Lab Chip*, 2004, **4**, 417-419.

[2] A. Iles, R. Fortt and A. J. de Mello, *Lab Chip*, 2005, **5**, 540-544.

[3] K. Jahnisch, V. Hessel, H. Lowe and M. Baerns, *Angew. Chem. Int. Ed.*, 2004, **43**, 406-446.

[4] D. J. Sadler, R. Changrani, P. Robert, C.-F. Chou and F. Zenhausern, *IEEE Trans. Compon. Packag. Technol.*, 2003, **26**, 309-316.

[5] J. El-Ali, P. K. Sorger and K. F. Jensen, *Nature*, 2006, **442**, 403-411.

[6] G. T. Roman, Y. Chen, P. Viberg, A. H. Culbertson and C. T. Culbertson, *Anal. Bioanal. Chem.*, 2007, **387**, 9-12.

[7] U. Seger-Sauli, M. Panayiotou, S. Schnydrig, M. Jordan and P. Renaud, *Electrophoresis*, 2005, **26**, 2239-2246.

[8] L. Gui and C. L. Ren, *Anal. Chem.*, 2006, **76**, 6215-6222.

[9] W. Rychlik, W. J. Spencer and R. E. Rhoads, *Nucl. Acids Res.*, 1990, **18**, 6409-6412.

[10] A. M. Chaudhari, T. M. Woudenberg, M. Albin and K. E. Goodson, *J. Microelectromech. S.*, 1998, **7**, 345-355.

[11] D. S. Yoon, Y.-S. Lee, Y. Lee, H. J. Cho, S. W. Sung, K. W. Oh, J. Cha and G. Lim, *J. Micromech. Microeng.*, 2002, **12**, 813-823.

[12] N. Fujisawa and Y. Hashizume, *Meas. Sci. Technol.*, 2001, **12**, 1235-1242.

[13] T. Nishikawa and H. Kambara, *Electrophoresis*, 1996, **17**, 1115-1120.

[14] M. A. Burns, B. N. Johnson, S. N. Brahmasandra, K. Handique, J. R. Webster, M. Krishnan, T. S. Sammarco, P. M. Man and D. Jones, *Science* 1998, **282**, 484-487.

[15] R. M. Guijt, G. W. K. van Dedem, N. F. de Rooij and E. Verpoorte, *Lab Chip*, 2003, **3**, 1-4.

[16] G. Y. Tang, C. Yang, H. Q. Gong, C. J. Chai and Y. C. Lam, *J. Phys.: Conf. Ser.*, 2006, **34**, 925-930.

[17] A. Dodge, G. Turcatti, I. Lawrence, N. F. de Rooij and E. Verpoorte, *Anal. Chem.*, 2004, **76**, 1778-1787.

[18] D. Ross, M. Gaitan and L. E. Locascio, *Anal. Chem.*, 2001, **73**, 4117-4123.

[19] J. Noh, S. W. Sung, M. K. Jeon, S. H. Kim, L. P. Lee and S. I. Woo, *Sens. Actuators, A*, 2005, **122**, 196-202.

[20] S. H. Kim, J. Noh, M. K. Jeon, K. W. Kim, L. P. Lee and S. I. Woo, *J. Micromechan. Microeng.*, 2006, **16**, 526-530.

[21] C. J. Easley, L. A. Legendre, M. G. Roper, T. A. Wavering, J. P. Ferrance, and J. P. Landers, *Anal. Chem.*, 2005, **77**, 1038-1045.

[22] N. Kitamura, Y. Hosoda, C. Iwasaki, K. Ueno and H.-B. Kim, *Langmuir*, 2003, **19**, 8484-8489.

[23] M. E. Lacey, A. G. Webb and J. V. Sweedler, *Anal. Chem.*, 2000, **72**, 4991-4998.

[24] M. E. Lacey, A. G. Webb and J. V. Sweedler, *Anal. Chem.*, 2002, **74**, 4583-4587.

[25] L. K. Davis, K.-L. K. Liu, M. Lanan and M. D. Morris, *Anal. Chem.*, 1993, **65**, 293-298.

[26] K.-L. K. Liu, L. K. Davis and M. D. Morris, *Anal. Chem.*, 1994, **66**, 3744-3750.

[27] S. Duhr, S. Arduini and D. Braun, *Eur. Phys. J. E*, 2004, **15**, 277-286.

[28] H. Mao, M. A. Holden, M. You and P. S. Cremer, *Anal. Chem.*, 2006, **74**, 5071-5075.

[29] Y. Sato, G. Irisawa, M. Ishizuka, M. Ishizuka, K. Hishida and M. Maeda, *Meas. Sci. Technol.*, 2003, **14**, 114-121.

[30] O. Filevich and R. Etchenique, *Anal. Chem.*, 2006, **78**, 7499-7503.

[31] K. G. Casey and E. L. Quitevis, *J. Phys. Chem.* 1988, **92**, 6590-6594.

[32] D. Ross and L. E. Locascio, *Anal. Chem.*, 2002, **74**, 2556-2564.

[33] J. Sakakibara, K. Hishida and M. Maeda, *Exp. Fluids*, 1993, **16**, 82-96.

[34] R. Samy, T. Glawdel and C. L. Ren, *Anal. Chem.*, 2008, **80**, 369-375.

[35] S. W. Magennis, E. M. Graham and A. C. Jones, *Angew. Chem. Int. Ed.*, 2005, **44**, 6512-6516.

[36] R. K. P. Benninger, O. Hofmann, J. McGinty, J. Requejo-Isidro, I. Munro, M. A. A. Neil, A. J. deMello and P. M. W. French, *Opt. Express*, 2005, **13**, 6275-6285.

[37] G. I. Redford, Z. K. Majumdar, J. D. B. Sutin and R. M. Clegg, *J. Chem Phys.*, 2005, **123**, 224504.

[38] R. K. P. Benninger, Y. Koç, O. Hofmann, J. Requejo-Isidro, M. A. A. Neil, P. M. W. French and A. J. deMello, *Anal. Chem.* 2006, **78**, 2272-2278.

[39] S. M. Matthews, A. D. Elder, K. Yunus, C. F. Kaminski, C. M. Brennan and A. C. Fisher, *Anal. Chem*., 2007, **79**, 4101-4109.

[40] R. K. P. Benninger, O. Hofmann, B. Onfelt, I. Munro, C. Dunsby, D. M. Davis, M. A. A. Neil, P. M. W. French and A. J. deMello, *Angew. Chem. Int. Ed.*, 2007, **46**, 2228-2231.

[41] Z. K. Petrasek, M. Krishnan, I. Monch and P. Schwille, *Microsc. Res. Tech.*, 2007, **70**, 459–466.

[42] S. Desportes, Z. Yatabe, S. Baumlin, V. Genot, J.-P. Lefevre, H. Ushiki, J. A. Delaire and R. B. Pansu, *Chem. Phys. Lett*., 2007, **446**, 212–216.

[43] T. Robinson, P. Valluri, H. B. Manning, D. M. Owen, I. Munro, C. B. Talbot, C. Dunsby, J. F. Eccleston, G. S. Baldwin, M. A. A. Neil, A. J. de Mello and P. M. W. French, *Opt. Lett*., 2008, **33**, 1887-1889.

[44] D.-A. Mendels, E. M. Graham, S. W. Magennis, A. C. Jones and F. Mendels, *Microfluid. Nanofluid.*, 2008, **5**, 603-617.

[45] S. Uchiyama, Y. Matsumura, A. P. de Silva and K. Iwai, *Anal. Chem*., 2003, **75**, 5926-5935.

[46] S. Uchiyama, Y. Matsumura, A. P. de Silva, K. Iwai, *Anal. Chem*. 2004, **76**, 1793-1798.

[47] K. Iwai, Y. Matsumura, S. Uchiyama and A. P. de Silva, *J. Mater. Chem*., 2005, **15**, 2796-2800.

[48] C. Gota, K. Okabe, T. Funatsu, Y. Harada and S. Uchiyama, *J. Am. Chem. Soc.*, 2009, **131**, 2766-2767.

[49] C. K. Chee, S. Rimmer, I. Soutar and L. Swanson, *Polymer*, 2001, **42**, 5079-5087.

[50] S. Uchiyama, T. Santa and K. Iwai, *J. Chem. Soc. Perkin Trans 2*, 1999, 2525-2532.

[51] C. Gota, S. Uchiyama, T. Yoshihara, S. Tobita and T. Ohwada, *J. Phys. Chem. B.*, 2008, **112**, 2829-2836.

[52] V. N. Haong, G. V. Kaigala and C. J. Backhouse, *Lab Chip*, 2008, **8**, 484-487.