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Resin Bead Micro-UV−Visible Absorption Spectroscopy

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The construction and design of a microscope coupled with a miniature UV−vis spectrometer is described. This was applied to the study of dyes linked to solid supports and displayed good correlation in spectral shape and λ_{max} values when compared to the dyes in solution, as well as showing a linear relationship between dye loading and UV−vis absorbance. The spectral profiles of these dyes at various pH's were measured and used to determine the pK_a of the dyes on the beads, which were compared with the pK_a values of the dyes in solution, thus enabling the dye-loaded beads to act as pH sensors.

The expansion of combinatorial chemistry has vastly increased the development of organic chemistry on solid supports,[1] and concurrently, a number of techniques have been developed for the analysis of compounds on these supports.[2−5] In the area of optical microspectroscopy, this includes Fourier transform infrared (FT-IR) microscopy[2,6] and single-photon, single-photon confocal,[7−9] and two-photon[10,11] fluorescence microscopy, as well as Raman confocal microscopy,[12−14] which have allowed the spatial mapping of the interior of a variety of supports, providing information regarding the functional site distribution within beads.

Colored beads have been generated for a number of other solid-phase and combinatorial reasons. Thus, a range of solid-phase strategies have been developed for the discovery and preparation of dyes,[15,16] colored beads have also been produced in a number of assays in which libraries have been screened for specific metal binding,[17] while host−guest chemistry has long exploited the localization of a dye-labeled guest onto beads containing a specific high-affinity host.[18] More recently, resin-bound indicators have been employed in monitoring the progress of solid-phase chemistries.[19,20]

In the case of dye synthesis, the ability to conduct UV−vis spectrometry on beads would enable a number of direct on-bead analyses to be carried out without requiring the additional step of isolating the chromophores. In the screening of receptors, on-bead quantitative measurements of spectrometric intensity would be a good indicator of the binding strength of the receptor. There are several advantages to the use of dyes over fluorophores in combinatorial screening; nonfluorescent dyes are generally cheaper and available in a wider variety of spectral and structural characteristics. Additionally, quantitative absorbance information of resin-bound dyes would be a step in the application of these materials as miniature sensor devices. Apart from this, the investigation of the colorimetric properties of indicator dyes on solid supports would provide insights into the physicochemical microenvironment within beads.

In this paper, the construction and design of a microscope used in conjunction with a miniature UV−vis spectrometer is reported, and initial testing and application in the study of dye and sensors linked to solid supports is described.

MATERIALS AND METHODS

UV−Vis Microscope Construction. A photograph and schematic plan of the combined microscope and UV−vis spectrometer system are displayed in Figure 1. The light source for the microscope and spectrometer was a Mini-D2T deuterium−tungsten fiber optic light source (Ocean Optics, Dunedin, FL) and the deuterium lamp had a spectral range of 200−410 nm, while

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(1) Nicolaou, K. C.; Hanko, R.; Hartwig, W. In *Handbook of Combinatorial Chemistry*: *Drugs*, *Catalysts*, *Materials*; Nicolaou, K. C., Hanko, R., Hartwig, W., Eds.; Wiley-VCH: Weinheim, Germany, 2002; pp 3−9.

(2) Gremlich, H.-U. *Biotechnol. Bioeng.* **1999**, *61*, 179−187.

(3) Dal Cin, M.; Davalli, S.; Marchioro, C.; Passarini, M.; Perini, O.; Provera, S.; Zaramella, A. *Farmaco* **2002**, *57*, 497−510.

(4) Gallop, M. A.; Fitch, W. L. *Curr. Opin. Chem. Biol.* **1997**, *1*, 94−100.

(5) Egner, B. J.; Bradley, M. *Drug Discovery Today* **1997**, *2*, 102−109.

(6) Haap, W. J.; Walk, T. B.; Jung, G. *Angew. Chem., Int. Ed.* **1998**, *37*, 3311−3314.

(7) Rademann, J.; Barth, M.; Brock, R.; Egelhaaf, H.-J.; Jung, G. *Chem. Eur. J.* **2001**, *7*, 3884−3889.

(8) McAlpine, S. R.; Schreiber, S. L. *Chem. Eur. J.* **1999**, *5*, 3528−3532.

(9) Moss, M. C.; Veiro, J. A.; Singleton, S.; Gregory, D. P.; Birmingham, J. J.; Jones, C. L.; Cummins, P. G.; Cummins, D.; Miller, R. M.; Sheppard, R. C.; Howard, V. C.; Bhaskar, N. *Analyst* **1993**, *118*, 1−9.

(10) Farrer, R. A.; Copeland, G. T.; Previte, M. J. R.; Okamoto, M. M.; Miller, S. J.; Fourkas, J. T. *J. Am. Chem. Soc.* **2002**, *124*, 1994−2003.

(11) Ulijn, R. V.; Brazendale, I.; Margetts, G.; Flitsch, S. L.; McConnell, G.; Girkin, J.; Halling, P. J. *J. Comb. Chem.* **2003**, *5*, 215−217.

(12) Kress, J.; Rose, A.; Frey, J. G.; Brocklesby, W. S.; Ladlow, M.; Mellor, G. W.; Bradley, M. *Chem. Eur. J.* **2001**, *7*, 3880−3883.

(13) Kress, J.; Zanaletti, R.; Amour, A.; Ladlow, M.; Frey, J. G.; Bradley, M. *Chem. Eur. J.* **2002**, *8*, 3769−3772;

(14) Kress, J.; Zanaletti, R.; Rose, A.; Frey, J. G.; Brocklesby, W. S.; Ladlow, M.; Bradley, M. *J. Comb. Chem.* **2003**, *5*, 28−32.

(15) Isacsson, J.; Westman, G. *Tetrahedron Lett.* **2001**, *42*, 3207−3210.

(16) Nguyen, T.; Francis, M. B. *Org. Lett.* **2003**, *5*, 3245−3248.

(17) Francis, M. B.; Finney, N. S.; Jacobsen, E. N. *J. Am. Chem. Soc.* **1996**, *118*, 8983−8984.

(18) Weingarten, M. D.; Sekanina, K.; Still, W. C. *J. Am. Chem. Soc.* **1998**, *120*, 9112−9113.

(19) Cho, J. K.; White, P. D.; Klute, W.; Dean, T. W.; Bradley, M. *J. Comb. Chem.* **2003**, *5*, 632−636.

(20) Cho, J. K.; White, P. D.; Klute, W.; Dean, T. W.; Bradley, M. *Chem. Commun.* **2004**, 502−503.

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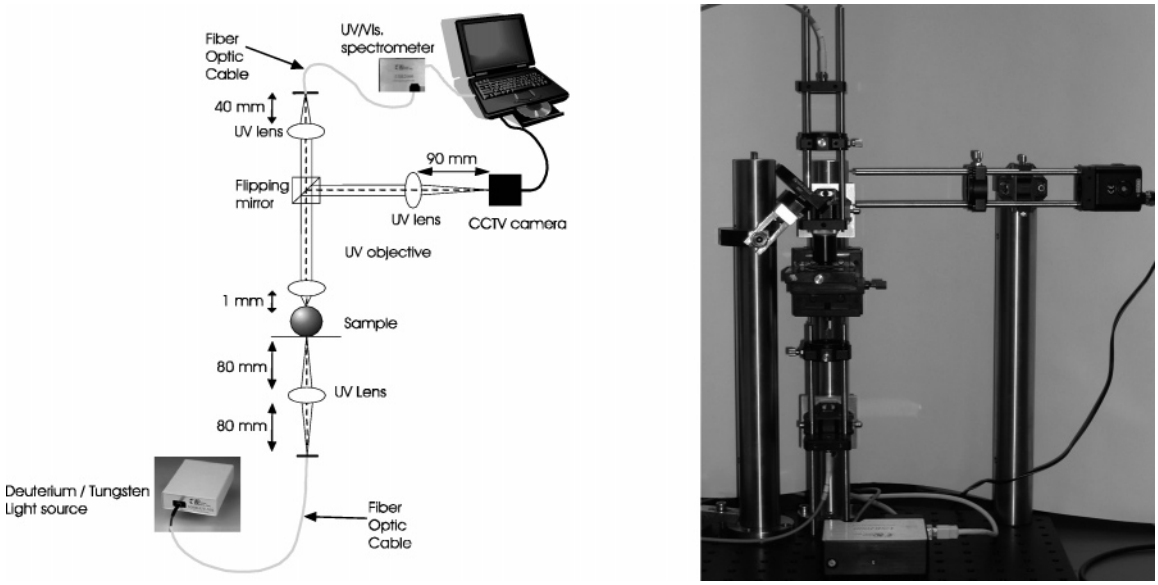

Figure 1. Schematic diagram of the custom-built UV−vis microspectrometer (left) and photograph (right).

the tungsten lamp covered 360−1100 nm (ignition delay < 2 s). A fused silica lens (40 mm focal length) was placed 80 mm from the light source, and the sample stage was located 80 mm above the lens. Above this was placed the objective lens, an OFR LMU-40×-NUV (numerical aperture 0.50) with a working distance of 1 mm and an effective focal length of 5 mm. The beam exiting the objective lens was then either refocused on to a fiber-optic cable leading to the entrance slit of a USB2000−UV−vis spectrometer (spectral range, 200−850 nm; typical overall spectral resolution, 1.5 nm; from Ocean Optics Inc.) or diverted to a closed circuit television camera using a flipping mirror. Another fused silica lens with a focal length of 40 mm was used to refocus the beam onto the fiber-optic cable. The magnification at the detector end was ×8 and at the camera end, ×18 while the microscope had a sampling area with a diameter of 25 μm. The spectrometer was connected to a PC which used OOIBase32 software (ver. 1.0.3, Ocean Optics Inc.) configured to measure UV−vis transmission intensity. The optic fibers used throughout had core diameters of 200 μm.

Materials and Equipment. Aminomethylpolystyrene (PS; PolymerLabs, Church Stretton, U.K.), TentaGel-S Amino[21] (TG; Rapp Polymere, Tübingen, Germany), aminopropyl-controlled pore glass (CPG; VitraBio, Steinach, Germany), *p*-methyl red (Tokyo Kasei Kogyo, Tokyo, Japan), *N*-Fmoc-3-nitrotyrosine (Advanced ChemTech, Louisville, KY), alizarin yellow GG (Sigma-Aldrich, Gillingham, U.K.), and mordant orange 1 (Sigma-Aldrich) were all used as supplied. (4-Carboxyphenyl)bromophenol blue[19] was donated by Dr. J. K. Cho (Centre for Combinatorial Excellence, University of Southampton). Dyes were coupled to the beads using standard amide coupling conditions. Full details of all the experimental procedures are given in the Supporting Information.

Solid-Phase UV−Vis Spectra Measurements. The acquisition time used was 1 s, and each processed spectrum was the average of eight different beads. The beads were soaked in the solution of interest for at least 1 h before being placed in a microscope slide well and the well filled with the solution. The measured transmittance spectra were then converted and further processed with the statistical software package Origin version 6.1 (OriginLab, Northampton, MA) to produce the absorbance spectra using Beer's law (Abs = $-\log(I/I_o) = \epsilon cl$). In the calculations, the concentration of the dye within the beads was used (c, was calculated from the known loading and volume of the beads) and the diameter of the bead was used for l.

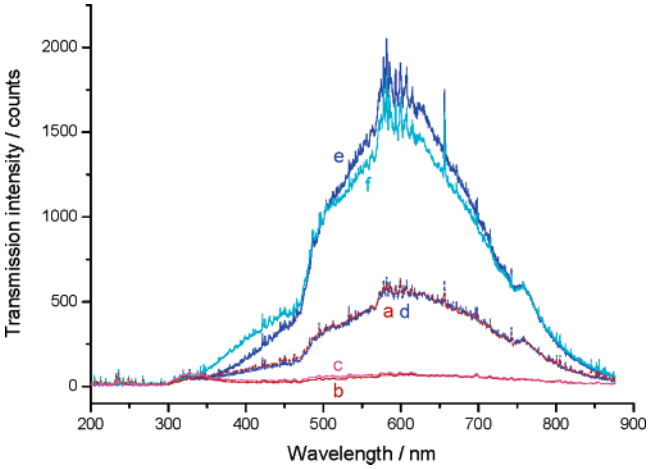

Figure 2. UV−vis transmission spectra of the unloaded beads: (a) glass slide only, (b) polystyrene dry, (c) TentaGel dry, (d) DMF on glass slide, (e) polystyrene swollen in DMF, and (f) TentaGel swollen in DMF.

RESULTS AND DISCUSSION

Preliminary Testing of the UV−Vis Microspectrometry System. Initially, the spectra of two types of beads were measured, aminomethyl-functionalized PS and poly(ethylene glycol) grafted polystyrene (TentaGel, TG). Using dry beads, reduced transmittance was observed since the opaque beads scattered the light. When swollen in solvent, beads typically swell up to 5 times their initial volume (volume of the swollen bead is ~90% solvent), and, as can be seen, the beads became "optically transparent" and allowed the transmission of light (Figure 2). Therefore, the work

(21) Rapp, W. *Methods Org. Chem. (Houben-Weyl)*, 4th ed.; Thieme: Stuttgart, Germany, 2002; Vol. E22a, pp 672−684.

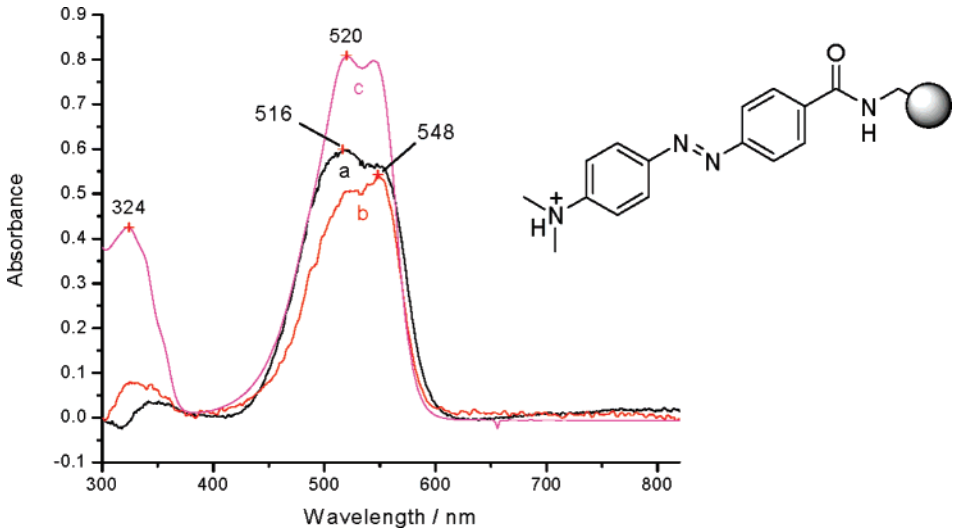

Figure 3. UV−vis absorption spectra of *p*-methyl red in 1.6 M HCl in 1,4-dioxane: (a) on PS (7% loading), (b) in solution in the microspectrometer (100 μM, 3 mm cell), and (c) in solution on a conventional spectrometer (25 μM, 10 mm cell).

presented here was carried out using only swollen beads in solvents.

An interesting feature observed in the initial testing was the apparent increase in the intensity of transmitted light through the swollen beads compared to the light beam passing through solvent alone. Above 400 nm there is approximately a 3-fold increase in the transmitted light due to the spherical beads acting as a lens and focusing the light into the collecting objective. Below 400 nm the absorption of the polymer support itself (or more accurately, the polymer and trace impurities within it from its manufacturing processes) reduces the transmitted light; this is a limitation inherent to working with polymer supports. A plot of the transmitted intensity through the bead in dimethylformamide (DMF) against the intensity through DMF alone for both PS and TG beads gave a near-linear relationship between 400 and 900 nm (with a slope of ~3; see Supporting Information for plots).

Solvents commonly used in solid-phase organic synthesis were evaluated to determine the extent of this effect and if it was possible to eliminate it. However, complete matching of the transmittance through the bead and through solvent alone was not possible using standard solvents. Depending on the optical properties of the solvent and its effect on the swelling of the resin, the lens effect was apparent with an increase in transmittance or with "poor" solvents; transmittance was reduced. To compensate for this effect, measurements were performed on swollen undyed beads and used as the reference. (See Supporting Information for the list of solvents used and results obtained.)

Qualitative UV−Vis. *Spectra of Solid-Supported Dyes.* Initially *p*-methyl red (Dabcyl) was coupled to amino-functionalized PS and TG resins and the UV−vis spectra of the beads were measured and compared with the dye in solution (Figure 3). The overall shapes of the spectra for the dye in solution and attached to the resins were observed to be similar although there was some alteration in the value of λ_{max}. This change may be the result of alteration of the chromophore by its attachment to the solid phase since this involved the conversion of the carboxylic acid to an amide. Additionally, the physicochemical environment around the chromophore was altered due to the presence of the polymer from the bead. A range of structurally diverse chromophores on various supports was then examined and compared with the dyes in solution, (4-carboxyphenyl)bromophenol blue[19] on TG, alizarin yellow GG on TG, 3-nitrotyrosine on CPG, and mordant orange 1 on TG, although larger differences in both the shape and λ_{max} of the spectra were observed. (See Supporting Information for full spectra.) Overall, the new system achieved good sensitivity with spectra being measured from beads with 10% or less of the functional sites loaded. A step further was then taken toward quantitative analyses.

Study of Dye Loaded Beads at Various Loadings. UV−vis spectra of PS beads, coupled with varying amounts of *p*-methyl red, were recorded and absorbance was plotted as a function of loading (Figure 4). Attempts were then made to quantify, in absolute terms, the relationship between loading and absorbance by applying the Beer−Lambert Law. The actual amount of dye coupled to the resins could not be accurately measured by either elemental analysis or other well-known tests due to the low loading levels involved (1−10%). To address this issue, the amount of coupled dye was determined indirectly through UV−vis analysis of the reaction solutions. Thus, for selected samples, the amount of dye in the reaction was measured pre- and postcoupling, and the difference was then used to calibrate Figure 4. By applying the Beer−Lambert law, ϵ_{max} for the dye attached to the beads was calculated to be ~7700 L mol^{-1} cm^{-1}. There was a similar linear relationship between loading and absorbance for (4-carboxyphenyl)bromophenol blue with a calculated ϵ_{max} ~ 8500 L mol^{-1} cm^{-1}. (See Supporting Information for full spectra.)

The values of ϵ_{max} calculated were significantly lower than those of the solution-phase analogues for both dyes (*p*-methyl red, 18 266 at 548 nm; (4-carboxyphenyl)bromophenol blue, 21 233 at 595 nm; in the same solvents as used for the beads). The reason for this is unclear, but ϵ_{max} may be affected by the lower polarity of the surrounding environment within the polymer matrix and the high concentration of dye within the bead and may also be the result of the previously mentioned "lensing" optical effect on the beads.

UV−Vis. *Profiling of pH Indicator Dyes.* *p*-Methyl red and bromophenol blue are used as pH indicators, and the pK_a of a dye in solution can be determined by UV−vis spectrometry.[22] To test the applicability of this on the solid-supported indicators, the

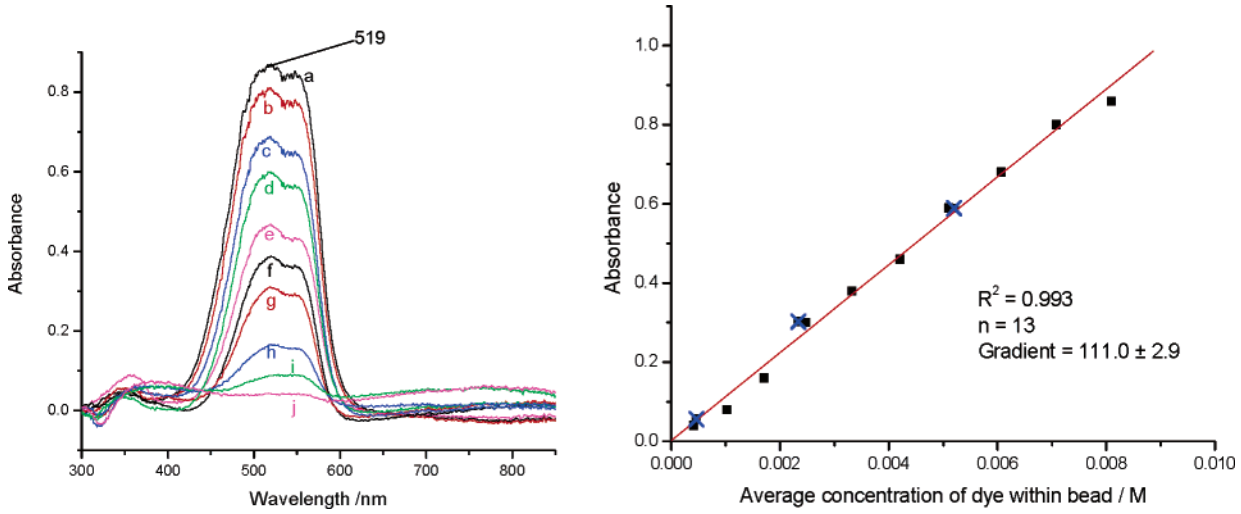

Figure 4. UV−vis absorbance spectra of *p*-methyl red PS beads at various loadings in 1M aqueous HCl in 1,4-dioxane: (left) with nominal loadings of (a) 10, (b) 9, (c) 8, (d) 7, (e) 6, (f) 5, (g) 4, (h) 3, (i) 2, and (j) 1%. Linear fit of absorbance vs loading (right) and samples quantified by analysis of reaction solutions (×).

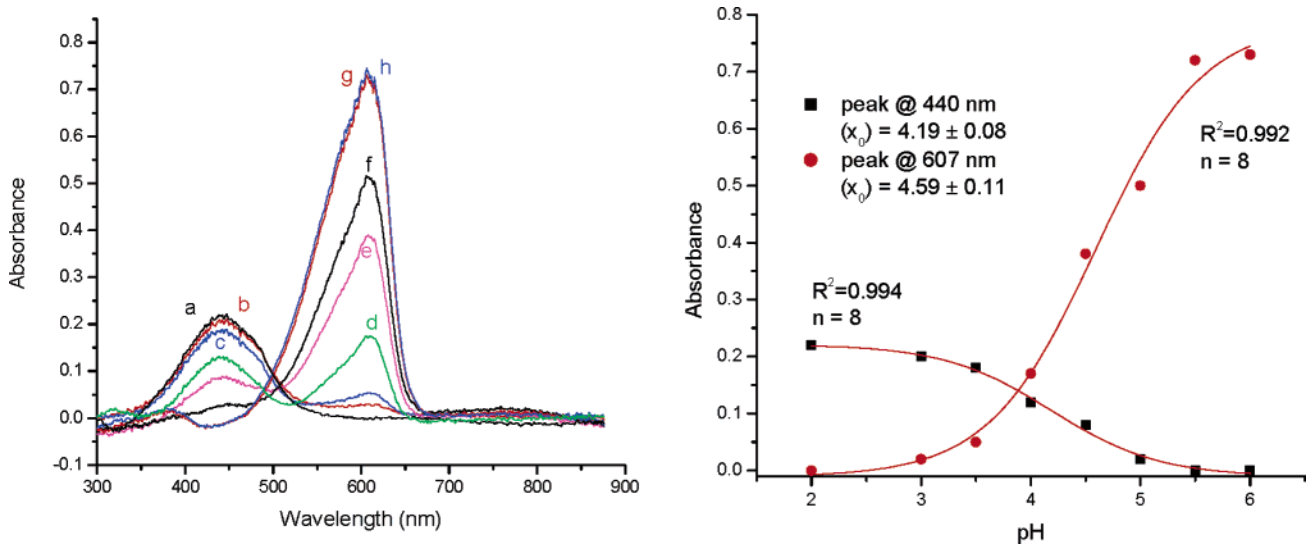

Figure 5. UV−vis absorption spectra of (4-carboxyphenyl)bromophenol blue beads in aqueous solutions (left) at various pH's: (a) 2, (b) 3, (c) 3.5, (d) 4, (e) 4.5, (f) 5, (g) 5.5, and (h) 6. Sigmoidal fit of absorbance vs pH (right).

spectra of (4-carboxyphenyl)bromophenol blue loaded on TentaGel (10% nominal loading) was measured at various pH's. The intensities of the two peaks corresponding to the acid and base forms of the dye were measured and plotted against pH with the best-fit Boltzmann sigmoidal curve (Figure 5).

From these plots, the pH at the midpoint between the maximum and minimum absorbance intensity (x_0) was determined. Thus, the pK_a from Figure 8 was estimated to be between 4.2 (based on measurements at 440 nm) and 4.6 (based on measurements at 607 nm).

For comparison, the spectra of a solution of (4-carboxyphenyl)-bromophenol blue were recorded at various pH's and the pK_a values obtained (3.94 for the peak at 440 nm and 4.29 for the peak at 594 nm) were very similar to literature values for unmodified bromophenol blue in solution.[22] Both were somewhat lower than for the resin-bound indicator, signifying that the deprotonation of the indicator on TentaGel required more basic conditions.

A similar experiment was conducted with TentaGel-linked *p*-methyl red (10% loading). The spectra in this case were more difficult to interpret due to the close overlap of the two possible absorbance bands so that in order to extract the desired information from these overlapping bands, deconvolution calculations were necessary. Using a model of two Gaussian peaks, the calculated maxima for these deconvoluted peaks (at 480 and 520 nm, respectively) were then plotted in a manner similar to the bromophenol blue derivative. (See Supporting Information for full spectra.)

From the plots, the estimated pK_a values in this case were 0.55 (from 520 nm) and 0.64 (from 480 nm). This was confirmed by visual observations of the color changes in this pH range. This differed significantly from the known pK_a of 1.78 for *p*-methyl red in solution.[23] To determine if amide bond formation may have been the cause of this, *p*-methyl red was coupled to methylamine. The solution-phase spectra of this modified indicator were then

(22) Escolar, D.; Haro, M. R.; Saucedo, A.; Ayuso, J.; Jimenez, A.; Alvarez, J. A. *Appl. Spectrosc.* **1996**, *50*, 1290−1294.

(23) Şeclăman, E.; Salló, A.; Eleneş, F.; Crăşmăreanu, C.; Wikete, C.; Timofei, S.; Simon, Z. *Dyes Pigm.* **2002**, *55*, 69−77.

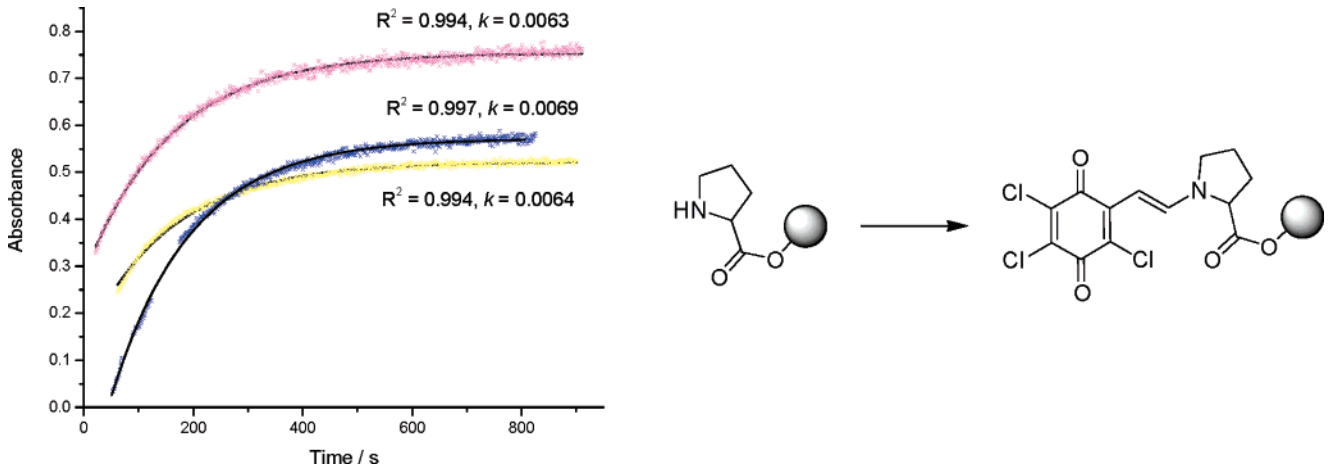

Figure 6. UV−vis absorbance at 550 nm plotted against time for three sample beads.

measured and the pK_a determined to be 1.66, which was again significantly different from the value for the solid-phase version. This implies that much higher H_3O^+ concentrations are needed to protonate the indicator moieties that are within the polymer matrix of TentaGel. This in turn could be related to the lower polarity of the matrix compared to a purely aqueous environment and the resulting lowered stability of the ionized forms of the indicators. Additionally, the high local concentration of indicator moieties within the bead and repulsion between the charged species may also affect their likelihood of protonation.

Changes in pK_a due to the altered environment caused by different solvents are well-known,[24,25] and changes due to the incorporation of indicators into various sol−gel matrixes have been described.[26−28] It would appear that alterations of pK_a can also be as a result of the local environment within a polymeric solid support commonly used in organic synthetic chemistry. Interestingly, such a great effect on pK_a was not observed with supported bromophenol blue when compared to *p*-methyl red.

On-Bead Colorimetric Reaction Kinetics. The use of the microspectrometer as a tool for real-time reaction monitoring was also investigated since its solid-state design and associated software enables spectra to be measured at a higher rate compared to split-beam spectrometers. Here, the chloranil test for secondary amines was studied where blue beads are produced in a pale yellow solution.[29] Proline, attached to 2-chlorotrityl-functionalized PS, was reacted with acetaldehyde, and a UV−vis spectrum of a single bead was measured every 1.0 s after addition of chloranil, and the absorbance at 550 nm was plotted against time (Figure 6). The λ_{max} value was not used in order to avoid saturating the spectrometer and to maintain linearity. Since the amount of acetaldehyde and chloranil used in each reaction was in excess, the only limiting factor was the number of amine sites on the beads, a pseudo-first-order reaction model could be applied. ($A_t = A_{max}[1 - e^{-k(t-t0)}]$, where A = absorbance at time t, A_{max} = maximum absorbance, k = rate constant, t = time, and t_0 = time when A is zero).

Overall, the data fitted very well with the model and the rate constant k demonstrated good reproducibility despite differences in bead size with an average value of 0.0065 s^{-1}, confirming that a reaction time of 5 min was sufficient for the positive determination of amines on-bead since the majority of the sites reacted within this time.

CONCLUSIONS

The UV−vis microspectrometer has proven to be a useful tool for the quantitative and qualitative measurement of a wide range of dyes on solid supports. Additionally, real-time rate measurements could also be conducted. This system can be employed regardless of the refractive properties of the solvent(s) or polymer support used and, in this respect, avoids the need to find index-matched solvents for each sample prior to analysis.

This spectrometer was used to investigate the effect of immobilization on the pK_a of indicator dyes, and significant differences were observed as a result of the attachment of the dyes to the beads. In both cases, ionization of the dyes required more extreme conditions, lower pH for protonation and higher for deprotonation. Nevertheless, these solid-supported indicator dyes continued to act as indicators, allowing beads to act as sensors of pH.

On-bead UV−vis microspectrometry can now be expanded to the study of solid-supported chromophores since the spectrometer is able to give quantitative values for colorimetric data which would have previously been simple qualitative (visual) examinations. Thus, a wide variety of chromophores such as sensors for pH or cations, or probes for various structural motifs on the solid-support, can now be explored.

SUPPORTING INFORMATION AVAILABLE

Table of relative signal intensity changes of beads in various solvents, detailed experimental procedures for the synthesis of dye-linked beads, *p*-methyl red methyl amide, and analysis of dyes and beads, and UV−vis spectra for solid-supported dyes, indicator dyes in solution, and pK_a measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) Lambert, J. B.; Shurvell, H. F.; Lightner, D. A.; Cooks, R. G. *Organic Structural Spectroscopy*; Prentice Hall: Upper Saddle River, NJ, 1998; pp 274−303.
(25) Gonzalez, A. G.; Herrador, M. A.; Asuero, A. G. *Anal. Chim. Acta* **1991**, *246*, 429−434.
(26) Rottman, C.; Grader, G.; De Hazan, Y.; Melchior, S.; Avnir, D. *J. Am. Chem. Soc.* **1999**, *121*, 8533−8543.
(27) Rottman, C.; Anvir, D. *J. Am. Chem. Soc.* **2001**, *123*, 5730−5734.
(28) Liang, C.; Weaver, M. J.; Dai, S. *Chem. Commun.* **2002**, 1620−1621.
(29) Vojkovsky, T. *Pept. Res.* **1995**, *8*, 236−237.