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PROPERTIES OF FLUOROPHORES ON SOLID PHASE RESINS; IMPLICATIONS FOR SCREENING, ENCODING AND REACTION MONITORING.

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Abstract: Fluorescent molecules covalently attached to resin beads are analysed to explore the application of on-bead fluorescence in combinatorial chemistry, and in particular: screening, encoding and reaction monitoring. The range of linearity of on-bead fluorescence is shown to be dependent on both the extent of loading and the Stokes shift of the fluorophore. Spectral deconvolution of multiple fluorophores on-bead is demonstrated. © 1997 Elsevier Science Ltd.

Combinatorial libraries, generated by solid phase organic synthesis, have rapidly become a major tool for drug discovery within the pharmaceutical industry. The technology allows the fast production of large diverse libraries and can lend itself to high throughput screening.[1] The detection, sensitivity and versatility of fluorescence has lead to its use in various aspects of combinatorial chemistry. An approach to combinatorial screening is by on-bead assays in which the fluorescent labelling of beads facilitates the identification of 'hits'.[2] Positive beads can be isolated manually, by the use of fluorescence activated cell sorting (FACS),[3] by fluorescence microscopy[4,5] or the compound can be cleaved and the fluorescence measured in solution.[6] Combinatorial libraries generated on spatially addressed modified glass slides have also been screened using fluorescence assays.[7] The use of fluorophores on solid supports may also be extended to encoding[8] and monitoring reactions on-bead.[9,10] Here, we report a study of the properties of fluorophores attached to polystyrene-based solid supports, and discuss the implications for on-bead screening and other applications of fluorophores in solid phase chemistry.

We have explored the fluorescence properties of a variety of commonly-used fluorophores attached to TentaGel (**a**) and aminomethylpolystyrene-1% divinylbenzene (**b**) at both low and high loading levels (Scheme 1). These labelling reactions proceeded in minutes and, for low levels of loading, yields based on the theoretical loadings were assumed. For higher loadings (>10% of the resin capacity), yields were confirmed by elemental analysis.[11] Three different techniques have been utilised to analyse the fluorescence of labelled beads: (i) single bead analysis *via* laser excitation, photomultiplier and broad band detector,[12] (ii) single bead analysis by a fluorimager scanner (fixed wavelength but allows many samples to be simultaneously processed), and (iii) analysis of a mono-dispersion of beads in a standard fluorometer.[13a-c] The three techniques were found to give consistent results however only the latter technique allows a full spectrum to be acquired.

The spectroscopic properties of fluorophores attached to polystyrene based resins at low levels (<1 pmol/bead) are very similar to their properties in solution. Fluorescence spectra of TentaGel (**a**) and polystyrene (**b**) labelled with fluorophores 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (**1**, NBD-Cl) and anthracence-2-sulfonyl chloride (**2**) respectively are shown in comparison to their solution spectra in Figure 1.[14] The excitation and emission bands, of the resin-bound samples, are not significantly shifted or broadened

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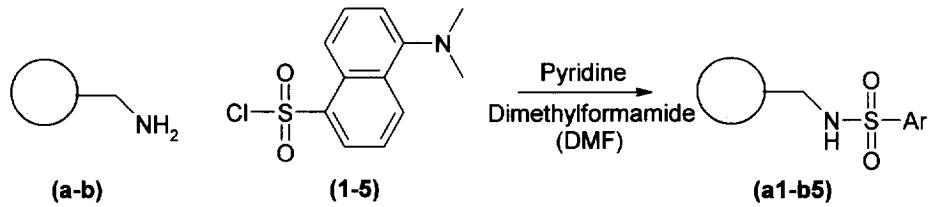
relative to the solution samples. Fluorescence spectra tend to be very environmentally dependent, therefore this result suggests an environment comparable to solution at such low loading. Fluorescence intensity was also found to be directly proportional to the amount of fluorophore loaded onto the resin (Figure 2). No self quenching or quenching of the fluorophore by the resin itself was observed at these levels of loading. These results suggest that polystyrene based resins have little effect on fluorophores of these types.



Scheme 1: (**a**) TentaGel resin (0.23 mmol/g), (**b**) aminomethylpolystyrene (1%DVB, 200-400 Mesh, 1.0 mmol/g), (**1**) 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), (**2**) anthracene-2-sulfonylchloride, (**3**) Texas Red® sulfonylchloride, (**4**) 5-dimethylaminonapthalene-1-sulfonylchloride (dansyl chloride), (**5**) 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF).

Extremely high sensitivity can be observed despite a low background fluorescence of many commercial resins. For example for loadings of Texas Red (**3**) on resin **a**, 10 fmol/bead (~0.01% loading) could easily be quantitated by single bead analysis.

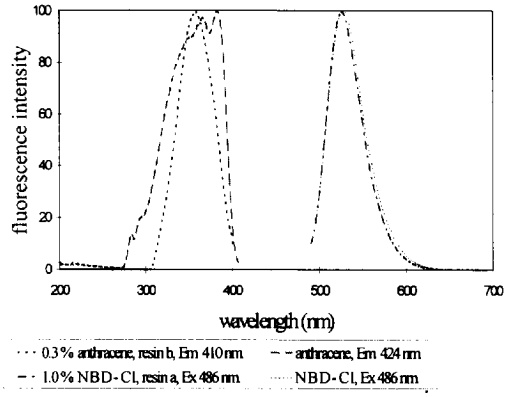


Fig 1: Spectra of resin bound fluorophores compared to their solution analogues. Solution spectra obtained of NBD-Cl (**1**) in a 5% solution of 2-methoxyethylamine in DMF and anthracene (**2**) in a 5% solution of benzylamine in DMF. Maximum intensities normalised.

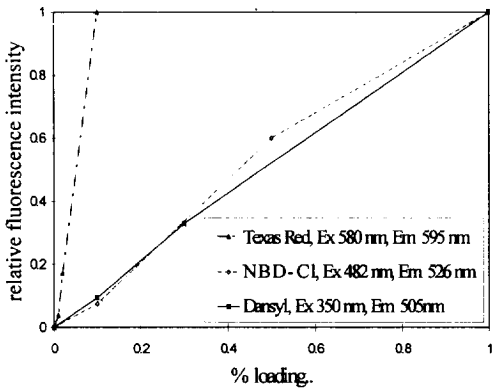


Fig 2: Resin **a** labelled with Texas Red (**3**), NBD-Cl(**1**) and dansyl (**4**). Analysis by laser,[13a] fluorimager[13b] and fluorometer[13c] respectively.

Resins can be simultaneously labelled with a variety of different fluorophores. TentaGel (**a**), labelled with low levels (<1%) of fluorophores: **1**, **2**, **3** and 5-dimethylaminonapthalene-1-sulfonylchloride (**4**, dansyl chloride), is shown in Figure 3. Since both excitation and emission wavelengths can be varied, overlapping bands between fluorophores is rarely a problem. When overlap does occur, peaks can be easily deconvoluted

using standard spectra of the individual fluorophores.[15] Since the fluorescence intensity of the labelled beads can also be accurately quantitated, beads can be loaded with different fluorophores at different levels giving even more variability. No quenching between fluorophores was observed at these low levels of loading.

To study fluorescence properties at higher loadings, the fluorescence intensity of TentaGel (**a**) labelled with varying amounts of 5-(4,6-dichlorotriazinyl)aminofluorescein (**5**, DTAF) was explored (Figure 4). Resin **a**, labelled with <5% of **5**, shows an approximately linear relationship between fluorescence intensity and the amount of fluorophore. Above a loading of 5%, however, the fluorescence intensity decreases dramatically. We attribute this to self quenching *via* fluorescence resonance energy transfer (FRET).

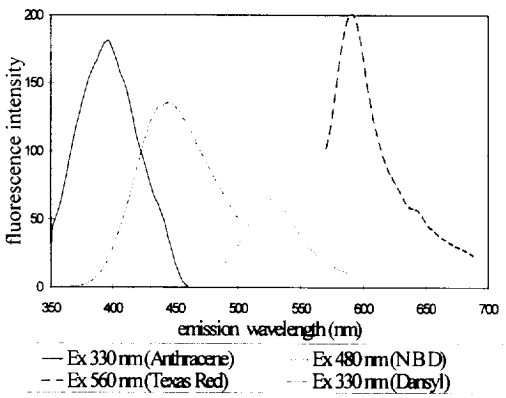


Fig 3: TentaGel labelled simultaneously with 0.1% Texas Red (**3**), 0.3% NBD-Cl (**1**), 0.3% dansyl (**4**) and 0.3% anthracene (**2**). Spectra of dansyl and anthracene have been deconvoluted. Absolute intensities.

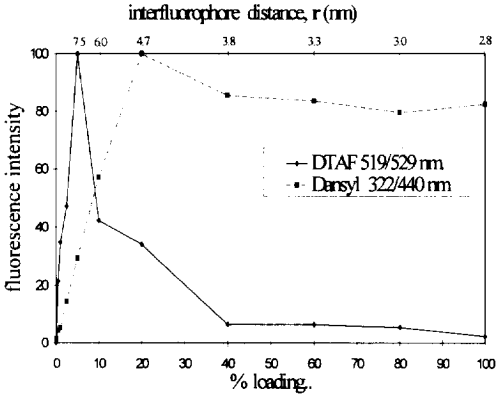


Fig 4: Quenching of resin **a** labelled with DTAF(**5**) and dansyl (**4**). Method: see ref. 12c. Inter-fluorophore distances calculated from the % loading (see ref. 16). Maximum intensities normalised.

The percentage loading of a resin can be converted to an average inter-fluorophore distance **r** within the swollen resin.[16] We can define a critical distance $\mathbf{r_o}$, where fluorescence intensity no longer increases with the concentration of the fluorophore. From the data shown in Figure 4, this was determined to be about 7.5 nm. Quenching of fluorophores on solid phase has previously been exploited using donor-acceptor systems, for on-bead protease assays, but self quenching has not been fully considered in these systems.[5]

Fluorophore	Resin	Relative Quantum Yield in Solution [17]	Stokes Shift (nm)	r_o (nm)
DTAF (**5**)	TentaGel (**a**)	0.68	10	7.5
NBD-Cl (**1**)	TentaGel (**a**)	0.47	44	6.2
Dansyl (**4**)	TentaGel (**a**)	1.00	118	4.7
Dansyl (**4**)	Polystyrene (**b**)	1.00	118	3.7

Table 1: Critical distances of fluorophores **1**,**4** and **5**, at which the fluorescence intensity of the fluorophore attached to a bead no longer increases with concentration. Quantum yields were measured in solution and may differ on resin.

All other fluorophores tested also showed the same quenching phenomenon, as observed with DTAF (**5**), at higher loading levels (Figures 4 and 5). It was anticipated that for a fluorophore with a large Stokes shift

(i.e. a large separation between peak excitation and emission bands), quenching should be reduced. Resins **a** and **b** were loaded with other fluorophores and the results are summarised in Table 1. A correlation between Stokes shift and $\mathbf{r}_0$ was observed and similar results were obtained with resin both resins.

Förster first described a critical transfer distance $\mathbf{R}_0$ at which the energy transfer rate (i.e. the degree of quenching between two fluorophores) is equal to the decay rate of the donor fluorophore.[18] Therefore $\mathbf{r}_0$ is a reasonable approximation of Förster's distance $\mathbf{R}_0$. This distance is dependent upon the orientation of the two fluorophores, the refractive index of the solvent, the quantum yield of the donor fluorophore, and the spectral overlap between the emission and excitation bands. If we again consider the two cases above, dansyl (**4**) and DTAF (**5**), their quantum yields are similar and both the average orientation of the fluorophores and the refractive index of the solvent can be considered to be constant. However, spectral overlap is much greater for DTAF which dominates in the observed increase in the value of $\mathbf{R}_0$. It can also be shown that the rate of energy transfer is proportional to $\mathbf{R}^{-6}$.[18] This explains why, as the concentration of the fluorophore increases, the transition at which significant quenching is first observed, is very sharp. Reported values for $\mathbf{R}_0$, for donor-acceptor systems, vary from 2 to 8 nm.[18] This is in excellent agreement with our approximate values of $\mathbf{r}_0$. However, a mathematical model of concentration quenching on resin is not explored here and other mechanisms which cause self quenching have been reported (e.g. π-stacking, dimer formation).[18]

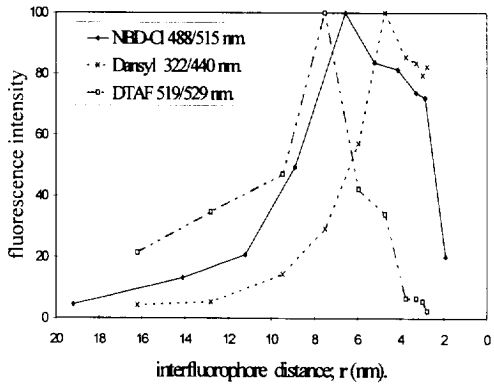


Fig 5: Fluorescence of resin **a** labelled with fluorophores **1**, **4** and **5**. Maximum intensities normalised. Methods: see ref. 12b, 12c and 12c respectively.

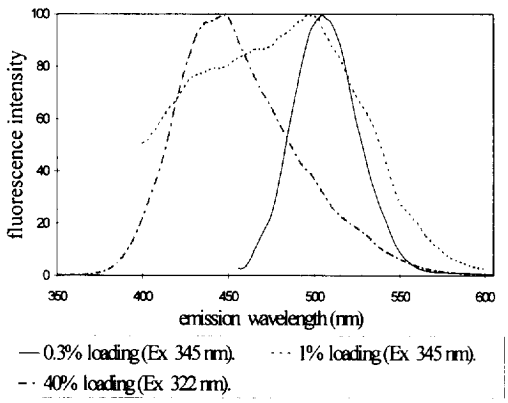


Fig 6: Blue shift in the emission spectrum of resin **a** labelled with dansyl (**4**). Maximum intensities normalised.

Band widths and band positions were found to alter as the level of loading increased. For resins highly loaded with dansyl (**4**), a blue shift in the emission band is observed (Figure 6). A shift was also observed with DTAF (**5**). These shifts are often reported in systems containing high concentrations of fluorophores. These collective observations have important implications for aspects of solid phase and combinatorial chemistries.

Combinatorial screening: These studies suggest that great care must be taken when carrying out on-bead combinatorial binding assays using fluorophores as probes. We have shown that there is a linear relationship between the amount of resin-bound fluorophore and the fluorescence intensity of a bead but that this relationship quickly breaks down as the amount of fluorophore increases and in most cases the fluorescence

intensity begins to decrease (Figure 4). Furthermore, the shape and position of spectral bands can also be dependent upon the amount of fluorophore. Most forms of commercial solid phase resins have, at best, limited accessibility to large proteins. Where these types of assays have proved successful, it is likely that the labelled probe only binds to the surface of the bead or the bead can only accommodate a relatively low level of internally-bound probe. In such cases, little self quenching would be observed, due to the low concentration, and consequently, a good relationship between binding affinity and fluorescence intensity has been reported.[3,7] However if the fluorophore is attached to a relatively high proportion of sites within the resin, then poor screening results can be expected. Work within our group to follow an enzymatic proteolysis reaction on solid phase by fluorescence staining of the resulting terminal amine gave poor discrimination in cases where the internal surface of the resin was accessible to the enzyme.[19]

Reaction Monitoring: A system where ninhydrin is used to quantitate the extent of a peptide coupling reaction on resin but a fluorophore is used to qualitatively assess the last 1% of the reaction has previously been described.[9] An attempt has also been made to measure the extent of Fmoc deprotection on resin by following the loss of fluorescence.[10] Work within our group to monitor solid phase reactions by fluorescence staining of resin bound unreacted groups followed by quantitative analysis is on-going. In a solid phase reaction unreacted amines on a single bead, for example, can be dansylated and the fluorescence of the resulting bead measured by one of the techniques described above. If we examine Figure 4, it can be seen that for the last 20% of unreacted amines (i.e. up to a 20% loading of dansyl (**4**)) the reaction extent would be inversely proportional to the fluorescence intensity of the bead, but between a yield of 0 and 80%, the fluorescence would be roughly constant. Therefore this approach could be applied to accurately quantitate single bead yields between 80 and 100% in some systems, which is very difficult by other techniques.

Encoding: An encoding strategy utilising fluorophores was reported by Yamashita and Weinstock,[8] The library is pre-encoded and beads are sorted by FACS machines after each round of the library synthesis, however the strategy was only demonstrated with two different levels of loading (10% and 100%) and with a single fluorophore. Such strategies must allow for self quenching if more levels are to be easily distinguished. We have shown that fluorescent molecules can be detected on-bead with high sensitivity (10 fmol/bead) and that the spectra resulting from multiple fluorophores can easily be deconvoluted on-bead (Figure 3). Quenching can be avoided by using much lower levels of the encoding fluorophores. Therefore in principle it should be possible to fluorescently encode beads using multiple fluorophores at different loading levels. The strategy allows the library to be pre-encoded which should be more cost and time efficient and have less problems with chemical orthogonality compared to other encoding methods. Also deconvolution does not require cleavage of either the library molecule or the encoding tags. Disadvantages include potentially poor chemical and photochemical stability of the fluorophores and the requirement for the entire library to be sorted by FACS machines. However, other strategies utilising fluorophores can be envisaged.

In conclusion, we have explored a range of properties of fluorescent molecules when covalently attached to resin beads. There is clearly scope to exploit the advantages of fluorescent molecules in various aspects of solid phase combinatorial methodologies. However, some care must be taken particularly where quantitative or semi-quantitative analysis is desired. These studies suggest that judicious choice of the fluorophore(s) and a

consideration of on-bead loading levels may enhance the design of certain combinatorial experiments.

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11. Typical labelling procedure. Dansyl chloride (**3**) (2.0 μmol, from a 0.50% stock solution in anhydrous DMF, 0.10 eq) and anhydrous pyridine (10 μl, excess) were added to resin **b** (1.0 mmol/g, 20.0 mg, 20 μmol, 1.0 eq) swollen in anhydrous DMF (1.0 ml). The solution phase lost its colour (yellow) within a few minutes while the resin became stained. The reaction vessel was sealed, excluded from light and allowed to stand at room temperature (3 hr). The resin was washed with DMF (2 x 2 ml), DCM (2 x 2 ml), MeOH (2 x 2 ml) and DCM (2 x 2 ml), and dried under vacuum. Yield was confirmed by sulfur analysis to within 10% of the theoretical yield.
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13. (a) Laser, Ex 527 nm, Em 580-650 nm. (b) Molecular Dynamics Vistra Fluorimager SI, argon-ion laser 488 nm, Em >515nm. The beads are spread out on a glass plate and scanned. Data points are taken from the average of 5 identically labelled beads. (c) Shimadzu RF 5001-PC spectrophotofluorometer. Spectra acquired from a mono-dispersion of resin, maintained by magnetic stirring, in dimethylformamide.
14. Solution analogues were synthesised *in situ* by acquiring the solution spectrum in DMF in the presence of 5% 2-methoxyethylamine or 5% benzylamine to mimic resins **a** and **b** respectively.
15. Spectra are deconvoluted by subtracting a weighted standard spectra of the first fluorophore such that a reference wavelength at which the second fluorophore does not emit is set to zero. Commercial software exists to deconvolute complex spectrum using methods such as 'least squares' if standards are known.
16. Resin **a**: loading 90 pmol/bead, size 90 μm, site dimension 1.9 nm (dry), 2.8 nm (3 times swollen in solvent). Resin **b**: loading, 175 pmol/bead, size 55 μm, site dimension 0.94 nm (dry), 1.4 nm (swollen). Resins purchased from Novabiochem.
17. Quantum yields of the 2-methoxyethylamine derivatives of the fluorophores were measured in DMF relative to the dansyl derivative. For methods see Bridges, J. W. *The determination of Quantum Yields, Standards in Fluorescence Spectroscopy*, Miller, J. N., Ed., Chapman and Hall: London, **1981**, 68-79.
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