Tagging in combinatorial chemistry: the use of coloured and fluorescent beads

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Fluorescent confocal microscopy is a practical and very sensitive (fmol bead^{-1}) and non-destructive tagging technique for use in combinatorial chemistry.

The power of solid phase and combinatorial chemistry is now being widely utilised by the major pharmaceutical players in the generation or optimisation of lead compounds in the drug discovery process.[1] Library generation can take place in a number of ways, perhaps the most powerful being the solid phase split and mix strategy such that each distinct 'bead' of the support contains only a single compound.[2] Screening from single beads requires means of compound identification and has led to the use of tagging, perhaps the most elegant approach being the arylcarbene system of Still.[3]

An addition to the area of tagging/decoding would be the use of the absorption or fluorescence spectrum of the resin bead. The simplest approach would be to covalently dye the beads and inspect under a microscope. The use of confocal fluorescence microscopy and complete spectrum analysis would enhance the sensitivity and potential of the system and enable the use of a number of fluorescent dyes which together with spectrum deconvolution would provide a very sensitive dye coding system.

In the first study a number of commercially available dyes† were covalently coupled by amide bond formation to TentaGel-S-NH_2 beads (130 μm) and a small peptide library (448 compounds $X_3X_2X_1$-linker-β-Ala-β-Ala-resin was prepared.[2] A selection of beads from the library were Edman sequenced and confirmed that the first residue (X_1) of the library was consistent with the colour of the single bead. The fluorescent microscope allowed ambiguities to be resolved, *e.g.* between pyrene and unlabelled TentaGel beads which were very similar by transmittance [Figs. 1(*a*)–(*c*)].

The fluorescence technique was extended by the use of a confocal microscope laser system.‡ The use of a single excitation line restricted the range of fluorescent dyes that could conveniently be used in these initial studies, however it was sufficient for initial proof of principle studies.

Single dyes–TentaGel beads (130 μm) were labelled with fluoroscein (0.01%) and erythrosin (0.02%).§ The beads were swollen in water, placed on a microscope slide with a cover slip and analysed under a confocal microscope‡ to give the spectra shown in Fig. 2. The spectra were clearly defined, relatively sharp and with distinguishable maxima. The degree of dye loading was varied by several orders of magnitude to give the spectra shown in Fig. 3. This showed the expected decrease in intensity with decreasing nominal bead loading and that as calculated increasing the bead loading above 0.1% did not give a uniform increase in relative fluorescence due to dye saturation. Fig. 3 shows loadings at 1 in 10^5 and demonstrates that even at this level of tagging the fluorescent signals are clearly observable, corresponding to approximately 3–4 femtomoles bead^{-1}. Labelling down to 1 in 10^6 (300–400 attomoles bead^{-1}) has also been demonstrated to be possible, although in this case the limits were determined by the amount of background fluorescence and Raman scattering from the bead

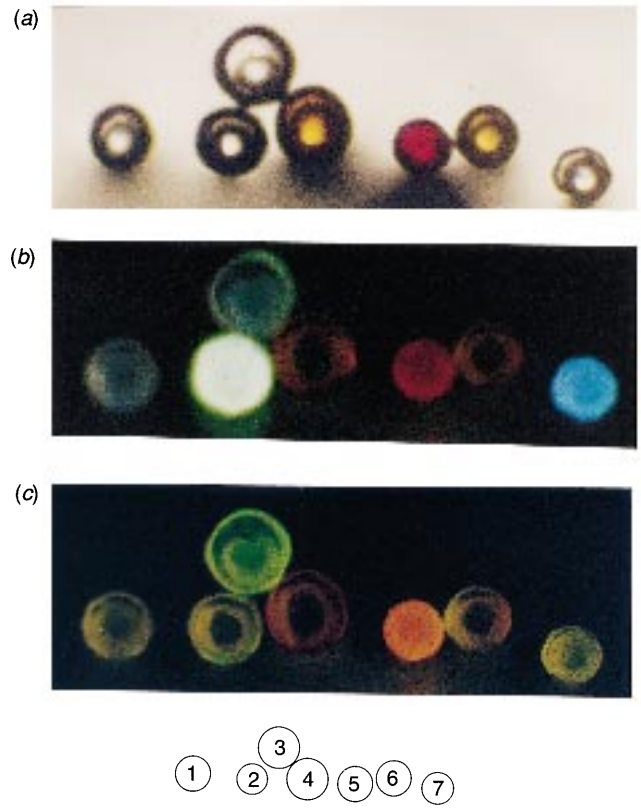

Fig. 1 Tagged beads under differing observation conditions 1 = unlabelled bead, 2 = dansyl, 3 = fluorescein, 4 = methyl red, 5 = erythrosin, 6 = mordant orange, 7 = pyrene. (*a*) Transmittance. (*b*) Fluorescence (excitation 340–380 nm, suppression filter 425 nm). (*c*) Fluorescence (excitation 420–490 nm, suppression filter 515 nm).

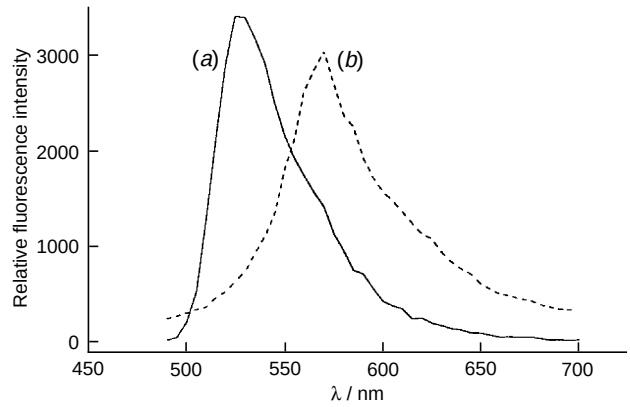

Fig. 2 Fluorescence spectra obtained from single tagged TentaGel beads. (*a*) 0.01% Fluorescein. (*b*) 0.02% Erythrosin.

itself. The number of molecules excited in this case is approximately 20 000. The intensity of fluorescence between beads of the same loading was also relatively uniform.¶

Multiple dyes–The potential problems associated with using several dyes in a tagging system is one of internal quenching by either energy transfer or reabsorption by the dyes. However, we reasoned that, due to the unusual environment created within a bead such quenching phenomena might be reduced in nature due to site isolation, especially at low substitution levels. Initial studies using beads with approximately equimolar numbers of dye labelled sites (0.01%) gave spectra which showed quenching was taking place but that it was not complete. The experiments were repeated keeping one dye at a fixed level within the beads and varying the ratio of the other. The spectra are shown in Fig. 4.

We have in this initial study used two very similar dyes with strongly overlapping absorbance and emission spectra and clearly this is a worse-case scenario. It does however suggest that, by using several different dyes with varying absorbance/emission spectra and by varying the dye loadings (by orders of magnitude), a viable tagging method can be produced.

The simplicity of using dyed beads to allow initial position identification has been demonstrated. If spectra are known for the various starting beads then the initial reaction component of the library when added to the bead is therefore known. The technique is non-destructive, thus allowing full subsequent analysis by other techniques, very sensitive, with detection levels easily down to femtomoles of material/bead and with a linear response to tagging levels. Although limited in this report, it will be simple to expand these studies by the use of a greater range of dyes with reduced reactivity. The use of coloured and fluorescent beads has the potential, we believe, to simplify the identification of library members for single bead screening applications.

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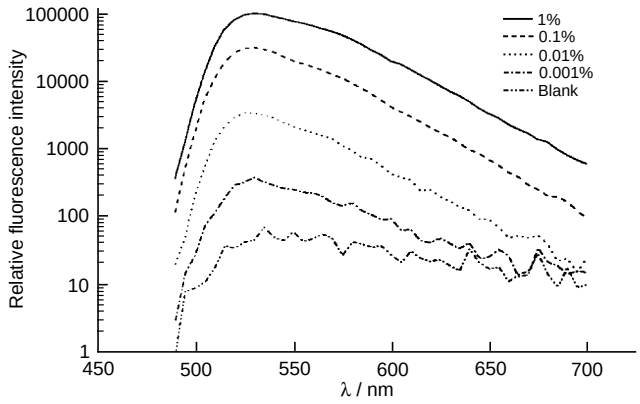

Fig. 3 Fluorescence linearity with loading. Relative fluorescence intensity against wavelength at varying tagging levels.

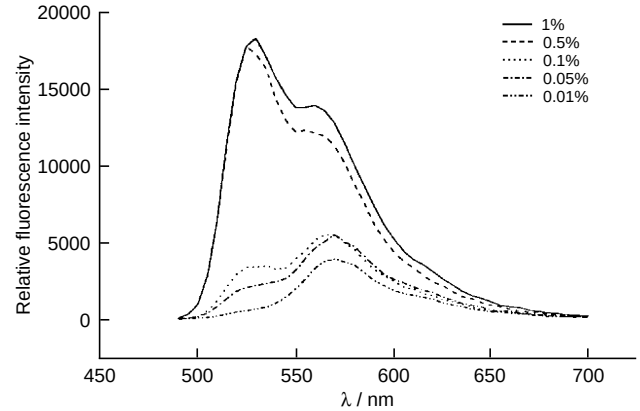

Fig. 4 Dye combinations. Fixed erythrosin level (0.02%) with varying levels of fluorescein (1, 0.5, 0.1, 0.05 and 0.01%).

Footnotes

† The dyes utilised in this study and the corresponding first amino acid in the library were pyrene butanoic acid (Val), methyl red (Ala), erythrosin B (Gln), dansyl chloride (Ser), fluorescein isothiocyanate, mordant orange (Asp) and succinylated disperse red (Leu), unlabelled beads (Lys). The following Fmoc amino acids were used in library generation: [X_1 = Ala, Asp(Bu^t), Gln, Leu, Lys(Boc), Ser(Bu^t), Val], [X_2 = Ala, Gly, Leu, Lys(Boc), Ser(Bu^t), Phe, Pro, Val], [X_3 = Asp(Bu^t), Gly, Leu, Lys(Boc), His(Trt), Ser(Bu^t), Phe, Val]. Four erythrosin labelled beads gave the amino acid sequences LAQ, HPQ(2) and GKQ, two pyrene labelled beads gave the sequences GPV and GFV, two methyl red labelled beads gave the sequences LPA and VQA and a mordant orange labelled bead gave the sequence GFD. TentaGel beads had a loading of *ca.* 300–400 pmoles bead^{-1} as determined by Edman sequencing.

‡ Fluorescence was excited using a focused Ar^+ ion laser beam with a wavelength of 476 nm and a power of approximately 4 μW. The approximate spot radius was 0.5 μm, giving a laser intensity of *ca.* 5×10^6 W m^{-2}. This was well below the saturation intensity of the dye (approximately 10^9 W m^{-2}) so all fluorescence should be linear in laser intensity. The area of the laser spot on the bead was *ca.* 8×10^{-13} m^2. The illuminated region of the bead can be approximated as a cylinder with a cross-section equal to the laser spot size and a length equal to the bead diameter. The illuminated volume, 1×10^{-16} m^3, is thus only 1 part in 11 000 of the total bead volume. Assuming the available binding sites (400×10^{-12} moles bead^{-1}) are evenly distributed throughout the bead, then there will be 2×10^{10} binding sites within the illuminated volume and an effective concentration of 0.33 m if all the sites are filled. Taking into account the dye absorption coefficient, the path length through the bead and the effective concentration, the Beer–Lambert Law absorption by the bead will be $I = I_0e^{-a}$, with $a = 370$. If only a fraction, f (filling factor), of the sites are loaded the absorption produced by the dyed bead is $I = I_0e^{-af}$. For occupancies above $f = 10^{-2}$ (*i.e.* > 1%) the bead will be optically thick and the exponential will depart significantly from a linear relationship between dye loading and fluorescent intensity—the addition of more dye will not significantly increase the fluorescence observed because all the available light is already being absorbed by the dye and so the addition of more dye produces no more fluorescence. The light was dispersed with a scanning triple monochromator and detected with a photomultiplier.

§ The beads were labelled by treatment with varying equivalents of FITC. The nominal % bead loading is the maximum number of sites which could be labelled under the reaction conditions. Beads were labelled with erythrosin using DIC and HOBt in DMF. Relative loadings for erythrosin were derived by comparison of the relative fluorescent intensities with FITC labelled beads and the extinction coefficients of the two dyes.

¶ The average fluorescent intensity of 12 beads (± S.D) at the nominal bead loading 1%, 123 000 ± 15 000; 0.1%, 27 400 ± 8000; 0.01%, 2950 ± 610; 0.001%, 271 ± 70.

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