Single Resin Bead Kinetics Using Real Time Fluorescence Measurements

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Received August 5, 2002

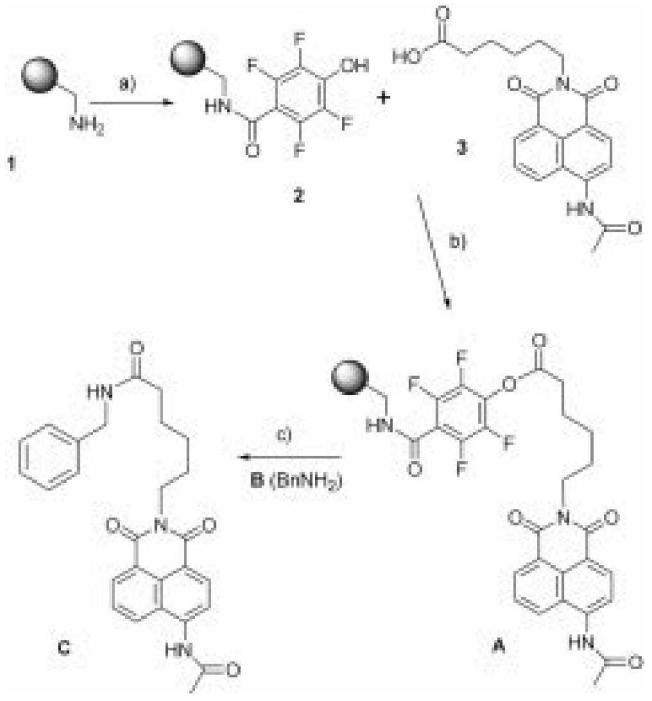
Key Words : Combinatorial library, Real time fluorescence measurement, Single resin bead kinetics, Solid phase labeling reagent

Fluorescence techniques have been used widely in chemistry and biology for over a century.[1] With the advent of combinatorial solid phase synthesis, a variety of fluorescence techniques for resin beads have been developed and adopted for broad applications, which include encoding/decoding,[2] fluorescent molecule binding assays,[3] and catalytic activity detection.[4] The split-and-pool method, another important technique in combinatorial chemistry, facilitates the synthesis of millions of molecules in a one-bead-one-compound library format; in principle, each single bead of resin acts as a separate microreactor. Although conventional screenings of these split-and-pool libraries have usually been performed as mixtures, a segregation approach of each single resin has been demonstrated recently, avoiding complicated data analysis.[5] To simplify the handling of a single resin, the researchers necessarily utilized a super size bead (a 500 μm diameter Rapp PS (polystyrene) bead, about 5-fold larger in diameter than an ordinary synthetic resin) with the aid of bead arraying tools.

The problem with the kinetics of solid phase reactions is that it demands, in many cases, if not all, tedious sample preparation steps including filtering, washing and transferring. While single resin bead FT-IR spectra have been utilized successfully to quantify solid phase reaction rates,[6] no comparable fluorescence kinetics has yet been demonstrated. Herein we report the first example of single resin bead kinetics using continuous measurement of fluorescence generation on an activated ester resin.

Activated esters on solid support have been developed as convenient labeling reagents, especially for amine nucleophiles.[7-9] Most of the reported functionalities, such as nitrophenol, N-hydroxysuccinimide, HOBt (1-hydroxybenzotriazole) and Kaiser oxime, have been attached to a polystyrene solid support by a Friedel-Craft reaction[10] or to a thiol resin by a maleimide linker,[9] limiting the selection of resin compositions. To overcome this limitation, we utilized the well-established amide bond formation to couple tetrafluoro-4-hydroxy benzoic acid with an aminomethyl resin (**1**), which has a broad material selection, creating a novel tetrafluorophenol resin (**2**).[11] For kinetic studies under various conditions, an environment-insensitive high quantum yield fluorescence molecule, 4-acetamino-1,8-naphthalimide (**3**),[7] was chosen to prepare the activated ester resin (**A**).[11] When reacted with a small amount of benzylamine (**B**) in various solvents, resin **A** gave a pure product (**C**) without work-up or purification, and the amount of generated product could be quantified by fluorescence measurement. Similarly obtained fluorescence data was previously used to calculate the second order reaction rate constant of nitrophenol resin, after removal of the resin by filtration followed by a sample dilution.[7]

One important observation was that the fluorescence was quenched almost completely in the resin-bound molecule, while the released product **C** showed a bright fluorescence; the fluorescence on the resin was less than 1% of that of product **C** in solution. Considering the fact that the fluorescence of a dye with a large Stokes shift was not self-quenched on the resin bead,[12] the quenching effect of resin **A** may be due to physical confinement of the dyes on to a small resin rather than due to a photochemical quenching. This phenomenon opened up the chance to study an *in situ* kinetic behavior of this resin without any sampling process, which facilitated data collection in a short time. Another improvement was achieved by changing the second order reaction of



Scheme 1. (a) tetrafluoro-4-hydroxy benzoic acid, DIC, HOBt, DMF; (b) **3**, DIC, DMAP, DMF; (c) **B**, DMF.

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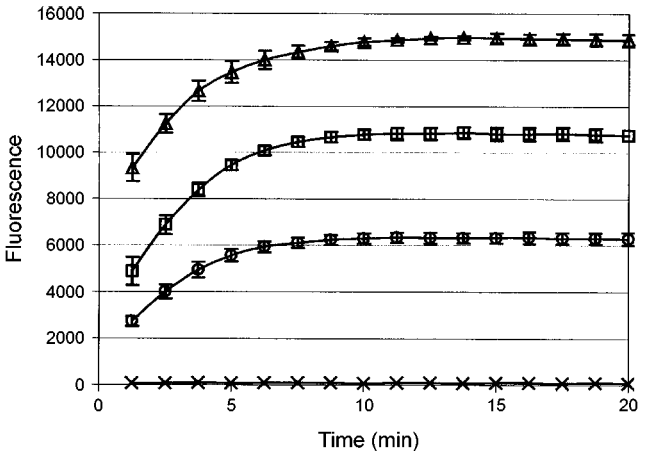

Figure 1. Fluorescence measurement of LCC PS resin (**A**) with $BnNH_2$ (10 mM) in DMF. x: no resin, circle: single resin, quadrangle: two resins, triangle: three resins. Standard deviation was given for the error bar.

aminolysis into a pseudo-first-order reaction by using an excess of the amine. This conversion has several significant advantages: 1) the kinetic reaction measurement time can be shortened from many hours (or even days) to less than one hour; 2) the pseudo-first-order reaction rate constant (k') can be obtained from the slope of a linear relationship of ln[1/1-relative conversion)] *vs.* time;[13] 3) although the kinetic starting point was not exactly synchronized due to an experimental deviation, it does not affect the calculation of k'; and 4) since the relative conversion, $[\mathbf{C}]/[\mathbf{A}_0]$, instead of the absolute amount of **A** consumed in the reaction profile, is utilized in the rate constant determination, measuring the initial amount of resin is not required. Normally one is obliged to use at least multi mg resins for each kinetic study. However, the last advantage allowed us to reduce the scale of the reaction, even down to a single resin bead.

To miniaturize the reaction, we tested a 384-well plate format with 50 μL reaction volumes containing benzylamine (10 mM final concentration), and recorded the fluorescence change using a plate reader (λ_{ex} = 370 nm, λ_{em} = 455 nm).[14] To make sure we were measuring a single resin bead's behavior, a homogeneous size resin (186 μm, LCC Reactospheres PS aminomethyl resin) was utilized. When diluted enough, zero to several resin beads could be transferred to the plate wells by pipetting and the fluorescence generation was recorded as a function of time as shown in Figure 1. While almost half of the wells showed no fluorescence, several wells showed a clear quantization of fluorescence generated from one, two, or three resin beads; there was no fluorescence curve intermediate between one and zero resin wells. A standard curve constructed with product **C** (1-100 μM in 50 μL DMF solution) indicated that the loading level of each single bead is about 1.1 nmol/resin (22 μM in 50 μL DMSO solution, the loading level of original aminomethyl resin is 2.7 nmol/resin).[15] Furthermore, the pseudo-first-order reaction rate constant k' was also calculated from a linear plot of the data, finally giving the second order reaction constant k as 46.9 ($\pm$ 5.5) $M^{-1}min^{-1}$. As expected, the resin number in each well did not affect the kinetic results.

In conclusion, we have successfully demonstrated the first single resin bead kinetics based on continuous fluoresecence measurement. This highthrouput kinetics approach will be useful to study the statistics of single bead behaviors and loading level measurements in a short period of time. The novel fluorescence labeling reagent containing tetrafluorophenol in various solid support materials will be used as key reagents to generate useful amide libraries and to label various amine-containing biological probes.

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14. Each resin (**A**) was initially suspended in dichloromethane and approximately 30 μL were transferred to each well of a 384-well plate. After the dichloromethane had completely evaporated, DMF (40 μL) was transferred into each resin-containing well and the resin was allowed to swell for approximately 5 min. Benzylamine (10 μL, 50 mM in DMF) was simultaneously transferred into each of these wells by a multi-channel pipette and the fluorescence was measured by a fluorescence plate reader (Spectramax GEMINI XS Microplate Reader, Molecular Devices). The excitation and emission wavelengths were 370 nm and 455 nm respectively, and the temperature was controlled at 25 °C. Each data point was recorded for intervals in the range of 20-120 seconds, and plate-shaking time was set at 5 seconds initially and 3 seconds between data readouts to ensure solution homogeneity.
15. Calculated loading level by the product company; personal communication with Dr. Willi Glettig of LCC Engineering & Trading GmbH (http://www.chemsupply.ch).