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Highlight

Analytical Techniques for Single Bead Analysis in Combinatorial Chemistry

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The demands of high-throughput screening (HTS) within the pharmaceutical industry have propagated the recent expansion of the field of combinatorial chemistry[1] and have led to a renaissance in solid phase synthesis. Combinatorial synthesis and library generation can take place in a number of ways, perhaps the most powerful and the most interesting to the analytical chemist being the 'split and mix' strategy in which synthesis is carried out on small polystyrene based beads in such a way that each distinct 'bead' of the support carries only a single compound [Fig. 1(*a*)]. Herein lies the power of the combinatorial methodology, since the number of compounds which can be generated is huge (potentially millions of compounds). However, it also provides some major challenges for the analytical chemist, for example, the ability to determine the structure of the compound on a single isolated bead from perhaps 10^5–10^6 structural possibilities. In addition, this must be achieved using beads of 100–200 μm in diameter and with only 200–400 pmol of material per bead. The second issue of concern for the analytical chemist is the monitoring of reactions carried out on the solid support. The advancement of combinatorial technology requires analytical methods capable of following organic transformations on solid supports rapidly, reliably and ideally in an automated manner. This Highlight will look at the most recent developments applicable to single bead analysis in both library synthesis and screening.

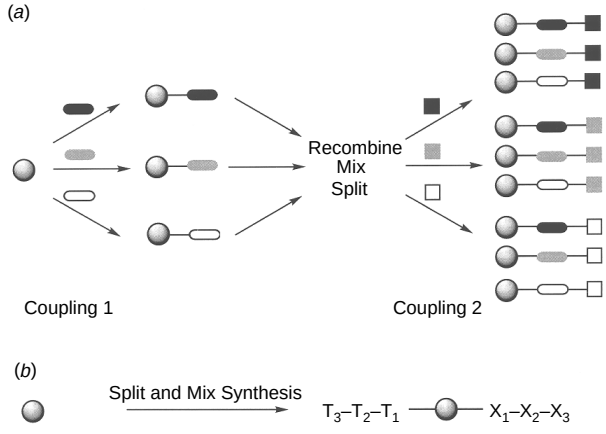

Fig. 1 (*a*) The 'split and mix' method of combinatorial library generation. Application of this methodology affords a single type of compound per bead. The power of this methodology is illustrated by the high products : reactions ratio. In this case, nine discrete compounds are generated. In general if resin beads are split x ways and subjected to n coupling steps, then x^n compounds are created in nx reactions. (*b*) The principle of tagging (encoding) in combinatorial library synthesis. Polymer bead: size, 50–200 μm, loading, 0.1–1 nmol sites/bead; functionalisation, Cl, OH, NH_2, *etc.* The library member is composed of three components ($\mathbf{X}_1$, $\mathbf{X}_2$, $\mathbf{X}_3$), and encoded for by a series of tags (T_1, T_2, T_3) which define both the composition and spatial location of the library component.

Encoding Strategies

An approach to determine the identity of the library component on the bead is to use a tagging system[2–3] which encodes the processes being carried out. Hence, the identification of the tag on a bead defines the synthetic chemical history of the bead and, hopefully, the structure of the active compound [Fig. 1(*b*)]. For a tagging strategy to be successful, a number of factors should be considered. These include the inertness of the tags to a broad range of chemistries, the simplicity of reading the tags from the bead and the ease of tag incorporation during the synthesis. There are a limited number of tagging strategies currently used in the area, all of which have major drawbacks.

Halogenated Aryl Tagging System

The attachment of a variety of derivatised halogenated phenols to the resin beads during a synthesis is a powerful, although perhaps synthetically cumbersome, method of tagging.[4–6] In the method described, a binary code of tags is utilised, with each tag being incorporated at a level of 1 pmol per bead after the synthetic step in question (although a more logical approach would be the use of pre-tagged beads and avoidance of the final mix at the end of the synthesis, thus simplifying the addition of tags to just one synthetic step). Tags are 'read' following single bead removal, oxidation, silylation and electron capture gas chromatography (ECGC) analysis [Fig. 2(*a*)]. This technique has been used in a number of single bead screening applications with success.[4–6] The advantage of this method is the inertness of the tags, which are well known in analytical chemistry. However, the extensive bead manipulation required to obtain the tagging information and additional synthetic steps in the synthesis are major drawbacks.

Peptide Tagging

Peptides have been used as a tagging method in combinatorial chemistry.[7–8] Peptide chemistry is robust and highly efficient, while peptide sequencing methodology is reliable and fairly routine in most biochemical departments. The amount of peptide needed for sequencing from beads is of the order of 5–10 pmol; thus there are more than sufficient sites on a single bead for tagging to a level of 5%. This technique has been used in a number of single bead screening applications with success [Fig. 2(*b*)]. The advantages of this method are the simplicity of the chemistry and the widespread availability of Edman sequencing. Importantly, no special bead manipulations are needed. The principal disadvantages are the restrictions in the range of chemistries due to the nature of the peptide chain.

DNA Tagging

The ability to amplify (PCR) and sequence DNA means that only tiny amounts of material are required as a code. This has

been used in one reported case to encode a peptide library on small (10 μm) beads.[9] However, although oligonucleotide chemistry is exceptionally reliable and fully automatable, DNA is not chemically robust enough to survive more demanding chemical syntheses. It is thus highly unlikely that such methodology will be adopted in future for encoding.

Amine Tags

An extension of the peptide tagging method has been reported by the Affymax group[10] in which a secondary amine tagging system was constructed on a secondary amide polymer, thus removing the potentially troublesome acidic protons of the peptide backbone. Tags were read following single bead removal, exhaustive acidolysis, simple derivatisation (*e.g.* dansyl chloride) and analysis by HPLC with fluorescence detection with a reported sub-picomolar sensitivity [Fig. 2(*c*)]. This technique has been used in a number of single bead screening applications. The advantage of this method is the inertness and availability of the tags but again it has the disadvantages of extensive bead manipulation to obtain the tagging information and the additional steps involved in the synthesis.

Dyes

Perhaps one of the simplest approaches to tagging is the use of fluorescent dyes and confocal microscopy, the method being non-destructive and offering remarkable sensitivity and decoding speed. There has been one report of this methodology in

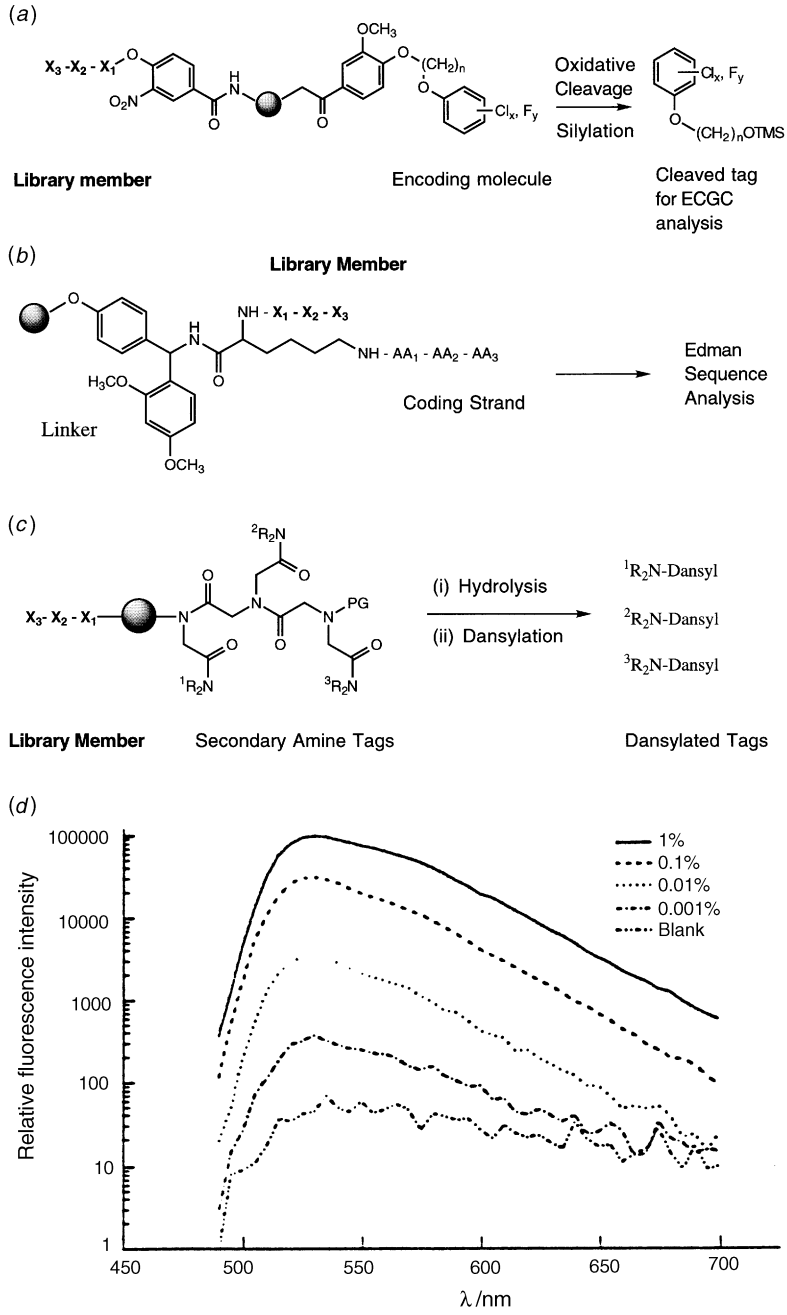

Fig. 2 (*a*) Synthesis of a combinatorial library encoded for by haloaromatic tags; (*b*) synthesis of a peptide-encoded combinatorial library. AA = amino acid; (*c*) combinatorial library encoded for by secondary amine tags. Once liberated, the amine tags are dansylated and then 'read' by HPLC; (*d*) relative fluorescence intensity of dyed resin beads against wavelength at varying tagging levels.

which a small peptide library was encoded at the first position by the incorporation of a dye [Fig. 2(*d*)].[11] The potential for use of several types of dye per bead and the problems of internal quenching were examined; due to site-isolation, the multi-dye approach was deemed a feasible method of analysis. The advantages of this method are the non-destructive nature of tag interrogation, the lack of chemical manipulations and its speed, with complete spectral analysis possible within seconds. There are also a wide range of tags available, some of which may be conveniently incorporated into the bead prior to synthesis. The lack of chemical inertness of the tags is a disadvantage, although the range of tags available may circumvent this problem as may the incorporation of tags into the bead structure.

Other Methods

It is envisaged that future coding strategies will simplify matters by utilising pre-tagged (MS or fluorescent/IR tags) beads at the beginning of the synthesis. Other methods,[12–13] although not single bead related, utilise capsules containing resin beads and microchip tagging devices.

Direct Analysis Strategies

There are a number of single bead analytical methods that have been used for the direct analysis of library related materials. The reader is directed to a recent review[14] for more general, non single bead analytical methodologies.

IR Spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a widely available, non-destructive technique, lending itself well to solid-phase analysis. The use of KBr discs of ground resin beads to record IR spectra was first reported by Frechet in the early 1970s, and has since been employed by others to monitor polymer supported syntheses.[15] Pivonka *et al*. have recently detailed the use of deuterium labelled protecting groups as highly characteristic IR signatures on the solid phase.[16] However, these methodologies are generally time-consuming and material inefficient (> 10 mg resin), and have lately been transcended by an array of superior sampling techniques.

Yan *et al*. introduced single bead FTIR microspectroscopy as a technique for the determination of product identity, reaction homogeneity and reaction time course on the solid phase.[17] An IR microscope was utilised to focus incident radiation upon an isolated resin bead (50 μm diameter Merrifield resin, 100 pmol of attached compound) to acquire data in either transmission or reflectance mode. The rate of progression of chemical reactions at the surface of a resin bead has been probed by attenuated total reflection (ATR) microspectrosocopy, an ATR objective being placed in contact with a single bead, thus flattening it slightly, and used to obtain an IR spectrum of primarily surface bound materials (radiation penetration depth ≈ 2 μm). Sensitivity is at the fmol detection level and thus offers the possibility of a tagging method for single bead analysis. Another form of reflectance acquisition IR is diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS). Sofia and co-workers[18] have illustrated that this technique is ideally suited to the high throughput on-bead monitoring of solid phase reactions; sample preparation is minimal, and resin can be analysed within 30 s. A third type of sampling mode has recently been employed in solid-phase reaction IR analysis, photoacoustic FTIR (PA-FTIR). An advantage of this method is that only the absorption component of the incident IR radiation contributes to the spectrum, thus the deleterious effects of light scattering, reflectance and resin non-homogeneity are nullified. This methodology was successfully applied by Lubell and co-workers.[19]

Continuous flow IR spectroscopy has also found application at the stage of solid phase reaction optimisation. This technique has been utilised to give real-time information on the progress of a variety of resin derivatisations.[20] Nielsen and co-workers have used near infrared (NIR) FT-Raman spectroscopy[21] to obtain information concerning the secondary structure of polyalanine peptides on TentaGel resins, although not on single beads.

Mass Spectrometry

Although inherently destructive by nature, mass spectrometry (MS) is guaranteed a pivotal role in solid phase analysis because of the rapid and detailed information it can provide, typically at the fmol level of the analyte. Three principal MS methods exist for direct bead interrogation: matrix assisted laser desorption/ionisation time-of-flight MS (MALDI-TOFMS), imaging time-of-flight secondary ion MS (TOF-SIMS) and electrospray MS (ES-MS).

Bradley and others have demonstrated that MALDI-TOFMS can be utilised to provide valuable information concerning a wide range of solid phase synthetic transformations on single beads. The technique involves *in situ* scission of the analyte-linker bond, before a matrix and calibrant are added, and the mixture is allowed to co-crystallise (5 min). Mass analysis is then effected by irradiation of the sample with an N_2 laser at 337 nm in a MALDI-TOF spectrometer. A diverse array of linkers have been shown to be amenable to this methodology.[22] Siuzdak and co-workers have employed Br-Wang linkers in their synthesis, allowing peptide to be photolysed *in situ* by laser radiation.[23] TOF-SIMS has likewise been used to follow the elongation and deprotection of polymer bound peptides.[24]

Youngquist *et al*. have reported a technique which permits the sequencing[25] of material from a single resin bead (Fig. 3) by a ladder synthesis approach *via* MALDI-TOFMS. At each coupling stage during the synthesis, a small proportion (10%) of a capping reagent is introduced, leading to a premature termination of a percentage of the library member. On cleavage

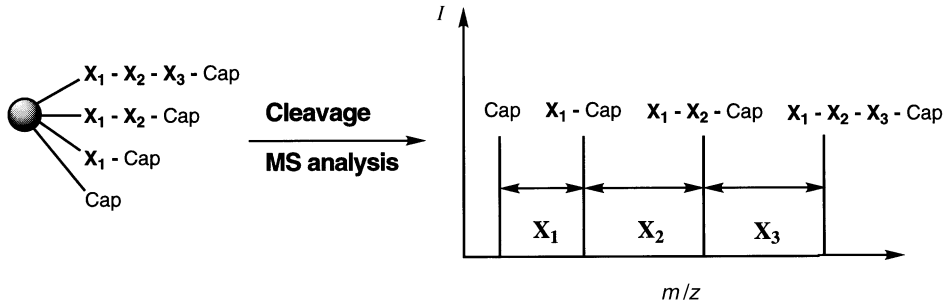

Fig. 3 Termination synthesis encoding. During the synthesis of $\mathbf{X}_1$–$\mathbf{X}_2$–$\mathbf{X}_3$ a small proportion of the sequence is capped at each stage. The identity of the library member is elucidated by 'reading' the MS ladder spectrum.

from the resin, MS analysis furnishes a peak for the full-length library member and a 'ladder' of truncated fragments. The sequence of the unknown is thus deduced from the mass differences between adjacent members of the termination synthesis family. Isobaric residues may be differentiated by use of orthogonal termination tags. The validity of this protocol was demonstrated by sequencing members from an acetylated peptide library, with a success of > 95%, at a rate of 25–30 residues per hour, using as little as 1% of the material from a single 88 μm bead.

A number of other mass encoding strategies have been outlined. For example, Geysen and co-workers[26] have described a methodology in which coding blocks (made up of various unlabelled and labelled, *e.g.*, ^{13}C, ^{15}N precursors) are used in varying ratios. However, it has not been used in library screening.

Brummel *et al*. have used the above mentioned three MS acquisition techniques in complement to analyse resin bound materials.[27] In each case, the parent ion was identified with sufficient precision (< ±0.01 Da) to eliminate candidates with similar mass. Valuable structural information was accessed from ESMS–MS and MALDI-TOF metastable ion MS for an angiotensin antagonist bound to a Sasrin linker. Different fragmentation mechanisms of the same molecule were observed by imaging TOF-SIMS. The small diameter of the Ga^+ ion beam used in this technique, allows materials on a single bead to be spatially resolved from beads bearing other library members. It is suggested by the authors that the adoption of Fourier transform ion cyclotron resonance (FTICR) MS will afford further improvements in high resolution mass assignments from resin bound ligands.

Rebek *et al*. have surveyed the population density of the 'molecular landscape' of small molecule sub-libraries by ESMS in both positive and negative ion format.[28] This two-dimensional approach led to the detection of the majority of library members. Isobaric residues could be discerned by collision induced dissociation (CID) followed by subsequent analysis of the daughter ion spectrum. Preliminary research indicates that the implementation of an on-line separation method, such as capillary electrophoresis with both positive and negative ion ESMS, would reduce mass overlapping of library members, therefore allowing more complex mixtures to be sampled.[29]

Conclusions

Although great strides have been made in the development of a range of analytical techniques for single bead analysis, there is huge scope for their improvement. The quest to screen materials from single beads is a major research programme in many pharmaceutical companies and research groups, and necessitates the development of a range of far more flexible and routine tagging technologies. It is in this area of research that the authors believe that considerable contributions await to be made by the analytical chemist. The addition of a host of new analytical methods to the existing repertoire is necessary to allow the full potential of solid phase organic chemistry to be realised.

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