Visualizing Functional Group Distribution in Solid-Support Beads by Using Optical Analysis

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Abstract: By using optical analysis to visually slice through fluorescently labeled ArgoPore, glass, polystyrene, and Tentagel beads, it is possible to establish where the interior functionalization sites occur within these four resins. By detecting the fluorescence emission of the fluorophore functionalized beads on cross sections of each bead, the position of the dye moieties, and hence the functional-group distribution, are observed. This powerful technique is useful not only in evaluating the polymerization and amino-functionalization processes, processes that are relevant to the reactivity of resins during solid-phase synthesis, it also examines the effective concentration of the functional groups throughout the bead. Thus, this technique will provide information that is useful when predicting resin reactivity and behavior during solid-phase synthesis reactions.

Keywords: combinatorial chemistry · fluorescence · optical analysis · polystyrene · solid-phase synthesis

Introduction

The use of polymer resins for solid phase synthesis plays an ever increasing role in synthetic chemistry.[1] Recent papers have described the benefits of a number of the most commonly used resins:[2] ArgoPore,[3] glass,[4] polystyrene,[5] and Tentagel.[6] Amino-functionalized resins, useful not only in solid-phase organic synthesis but also for attaching linkers and spacer arms,[7] are synthesized through either a post-polymerization functionalization process (typically a Friedel–Crafts alkylation),[8] or by using a copolymerization process[9] that incorporates the functionalized monomer during polymerization.[5] The distribution of amino sites may vary based on the functionalization process. Most efforts in examining the properties of solid supports focus on surface area, porosity, and swelling of the beads, and relatively little attention has been given to the site of functionalization, for example, diffusion and solvation within the microdomain of a bead. The observed heterogeneity of microdomains within a bead can yield important consequences for the macroscopic properties (such as swelling),[10] and can lead to nonhomogenous reactivity of functional groups within regions of a bead.[11]

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In this report the optical analysis technique, commonly used for analyzing biological structures,[12] is used to analyze the amino functional-group distribution of four types of beads: ArgoPore, glass, polystyrene, and Tentagel. This technique allows for visual cross-sectioning (slicing) through fluorophore-labeled beads, in which the dye moieties are covalently attached to the bead's amino termini. By detecting the fluorescence emission of the fluorophore on each bead cross section, the distribution of the dye moieties, and hence the amino functional groups, are seen. Use of this technique for the first time on beads, optical analysis provides a clear, concise, and accurate picture of the spatial distribution of the amino functional sites within a bead. This evaluation may allow assessment of both the polymerization and amino-functionalization processes, processes relevant to the reactivity of the resins during solid-phase synthesis.

Several studies have examined the functional-group distribution within polystyrene and Tentagel beads. These techniques include time of flight secondary ion mass spectrometry,[13] fluorescence-quenching experiments,[9b, 11] autoradiography,[14] and scanning electron microscopy.[10a, 15] These techniques, however, are limited when looking at important issues, for example, functional-group location within a bead and their access to reagents. Optical analysis, to be differentiated from optical photographs that show only surface morphology,[10a, 13] uses of mercury lamp as the light source to excite a given fluorophore that is covalently attached to the bead. Detection at the wavelength-specific emission of the fluorophore allows visualization of the dye distribution. Unlike confocal analysis,[11c] the detector collects the fluorophore's emission from the bead directly from the point source

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as well as emission from the first-order diffraction pattern. This allows relatively opaque objects to be visually sliced as the focal plane is moved along the z axis. The airy disk created from the diffraction pattern is seen as a blurred picture of each slice. By using a mathematical algorithm, the original position of the light is determined. This image provides a picture showing the functional site distribution through relatively opaque beads.

Two types of macroporous beads were examined by using optical analysis (Table 1): ArgoPore and glass. Both resins are rigid, nonswelling beads that have clearly defined pores within the bead and a high internal surface area, and were functionalized with amino groups after the manufacture of the beads. ArgoPore is a highly crosslinked polystyrene (20%) with a relatively high loading of functional groups per bead.[2a, 3] Porous glass beads, although frequently used in oligonucleotide[16] synthesis, have been neglected in their use as solid supports for organic synthesis owing to the low loading (typically 0.04 mmol g^{-1} observed with most commercial sources). However, recently porous glass beads with a loading of up to 0.3 mmol g^{-1}[4] (comparable with that of Tentagel) have become available.

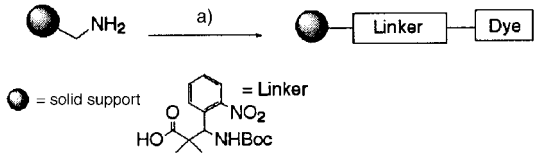
Table 1. Shown here is the A) theoretical loading of the beads directly from the manufacturer, B) theoretical loading after coupling with the linker and rhodamine dye, C) percent nitrogen found in the elemental analysis, and D) percent coupled to completion with the linker and dye (determined from the elemental analysis and theoretical loading)

	A [mmol g^{-1}]	B [mmol g^{-1}]	C [%]	D [%]
ArgoPore	0.74	0.49	3.11	90
glass	0.1	0.068	0.43	70
polystyrene	0.6	0.425	2.38	80
Tentagel	0.29	0.214	1.06	70

Two gel-type resins were chosen for optical analysis (Table 1): polystyrene (functionalized with amino groups after the polymerization process)[8] and Tentagel [amino functionalization is on the termini of the poly(ethylene glycol) (PEG) spacer arms]. Polystyrene resin, the most commonly used for solid-phase organic synthesis,[1, 17] contains styrene polymerized with 1–2% divinylbenzene. The low levels of crosslinking allow the resin to swell in numerous solvents (e.g., dimethylformamide, dichloromethane, and tetrahydrofuran). However, the crosslinking is thought to be nonuniform, with dense aggregate areas dispersed within lighter cross-linked regimes yielding heterogeneity within the bead.[10] Tentagel is approximately 70% (by weight) PEG that is grafted onto a polystyrene core. While high levels of PEG are required to achieve swelling with a larger range of solvents than polystyrene alone, it leads to low loading.

Results and Discussion

As the solid-phase beads are not inherently fluorescent a fluorophore was attached to the amino sites on the bead in order to visualize these sites by means of optical analysis. We attached rhodamine dye to each of the four types of amino-functionalized beads (Scheme 1). Thus, amino moieties should have a rhodamine molecule attached, allowing the site to be visualized by excitation of the dye at 540 nm and collection of emission light at 570 nm.



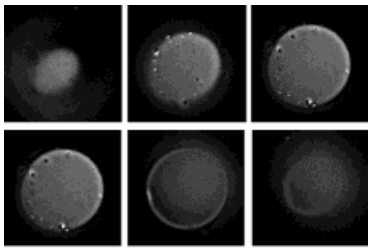
Scheme 1. Synthesis of fluorescent beads. a) i) linker, HATU (4 equiv), DIPEA (8 equiv), DMF/CH_2Cl_2. ii) TFA. iii) DMF/DIPEA, 5-(and 6-)-carboxytetramethyl Rhodamine dye (3 equiv).

As the beads gain mass with each successful reaction, the theoretical loading will decrease. The fluorophore-coupled beads were submitted for an elemental analysis and the nitrogen percentages that were found are shown in Table 1. By comparing the theoretical nitrogen percentage for a given mass of beads when fully coupled with linker and dye with the percentage found in the elemental analysis, the percent coupled to completion is obtained. These percentages suggest that complete coupling is achieved and that most or all amino termini initially on the bead are functionalized with linker and dye.[18]

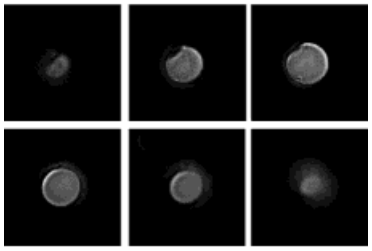
The optical analysis technique was used to obtain the optical slices of each bead type shown in Figure 1. While visually slicing through the beads, the clarity of the bottom portion of each bead demonstrates the utility of this method for relatively opaque beads. The reasonably consistent dispersion of fluorescence throughout the slices for ArgoPore and glass beads suggests that the reagent diffused completely throughout the bead during the functionalization processes and coupling. The relatively uneven distribution of dye sites on polystyrene and especially Tentagel shows a more congested environment in which the functional groups are clustered on the surface. These images reflect both the functionalization process for these four resins (all are functionalized post-synthesis) and the inherent properties of these beads.

Plotting the fluorescence intensity as a function of the bead diameter in each bead cross section illustrates clearly the differences between the bead types (Figure 2).[20] As successive slices of each bead are examined for fluorescence intensity, the dye distribution is relatively uniform throughout the ArgoPore and glass beads, but significant intensity differences are seen across the diameters of the polystyrene and Tentagel slices.

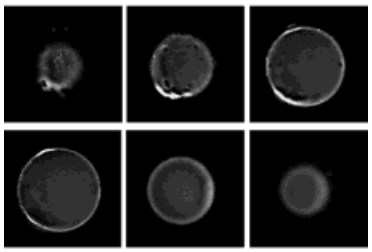
The intensities of the ArgoPore and glass cross sections show the relatively even distribution of dye molecules throughout the diameter. Although a small amount of clustering appears in the ArgoPore, only 14% of the dye is on the outer surface, with glass averaging 9%.[20] This suggests that the functionalization processes produce evenly functionalized beads and implies a uniform microdomain environment. Although ArgoPore and glass do not visibly swell with solvent, this analysis demonstrates that reagent diffusion occurs throughout the bead.



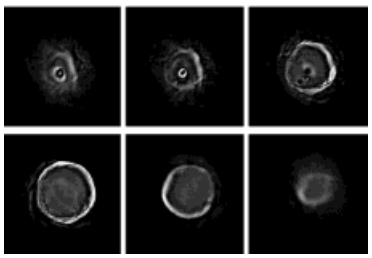
A) ArgoPore



B) Glass

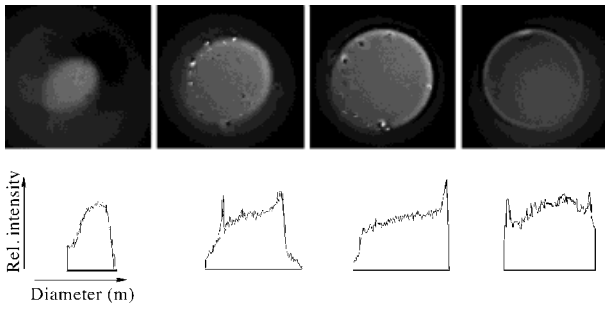


C) Polystyrene

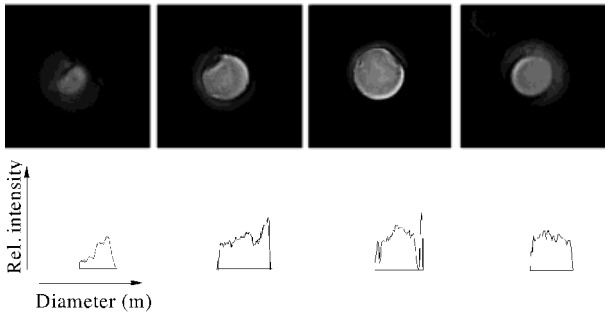


D) Tentagel

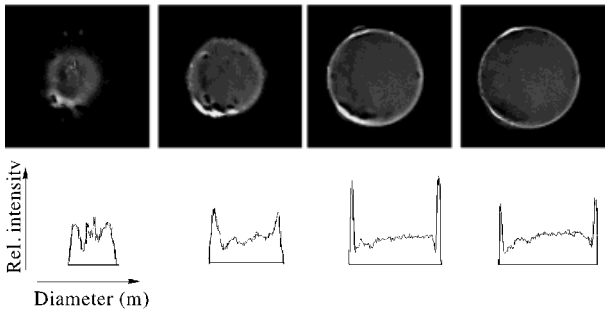
Figure 1. Four bead types were examined by optical analysis. A), B), C), and D) show optical slices of ArgoPore, glass, polystyrene, and Tentagel, respectively. Only one slice every 20 microns is shown. The slices at the top and the bottom of the bead are smaller than the center slices owing to the logistics of slicing a round object.[19]



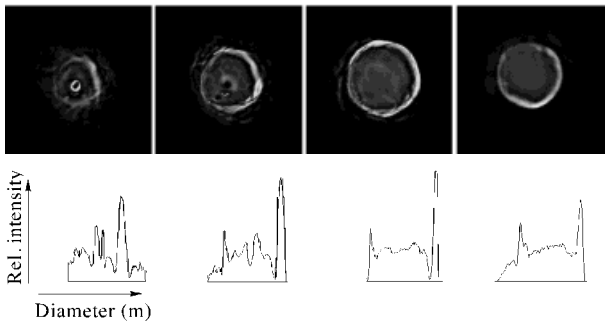
ArgoPore



Glass



Polystyrene



Tentagel

Figure 2. A graphical representation of the fluorescence intensity across the diameters of four consecutive slices of ArgoPore, glass, polystyrene, and Tentagel beads are shown and illustrate the significant differences of the dye distribution between the different resins.[21]

In contrast to ArgoPore and glass beads, polystyrene and Tentagel beads have a significant portion of dye sites (polystyrene 30%–35%; Tentagel 40%–60%) located on the outer surface.[20] Diffusion of reagents through both polystyrene and Tentagel appears limited as the outer surface was functionalized to a disproportionately greater extent than the center of the resin, with the core of Tentagel appearing significantly less functionalized relative to its outer surface than seen with the other three resins.[22]

The cross section of the top of a polystyrene bead is similar to that seen for glass and ArgoPore (compare the first slice for all three beads shown in Figure 2). The next two consecutive cross sections of these three resins differentiate polystyrene from glass and ArgoPore. Thus, unlike ArgoPore and glass, as sequential cross sections of the polystyrene bead are taken, the differential in the dye percentage between the outer 10 microns and the inner 70–80 microns increases and a 5–10 μm "shell" of functional groups are seen on the surface of the bead. This trend observed with polystyrene is also seen with Tentagel, although Tentagel beads have nonuniform surfaces that contain hollow cavities (see Figure 2 Tentagel

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cross sections) making each cross section significantly more irregular than the cross sections of a polystyrene bead.

The optical analysis results are consistent with the observation that an enzyme can cleave up to ~80% of a compound from a Tentagel bead and support the hypothesis that a significant portion of the compound is on the outer surface of the bead.[23] Several observations, which include the fact that the interior of the bead has a slower reaction rate than the outer surface[24] and that functionalizing polystyrene to higher capacities requires longer reaction times and a large excess of reagents,[8b, 8c] allude to functionalization occurring preferentially on the outer surface. This creates a congested environment, and the least accessible sites (interior) of the polystyrene become functionalized only after forcing conditions are applied.

Fluorescence quenching and trapping may occur during the fluorescence excitation and emission detection process of this technique. However, optical photographs in the absence of the fluorescence excitation provide evidence that these two phenomena have little or no effect on the optical analysis results (Figure 3). The dark red seen on the rims of the polystyrene and Tentagel beads show the significantly higher concentration of dye on the rim of these beads relative to their core, which is consistent with the fluorescence data.

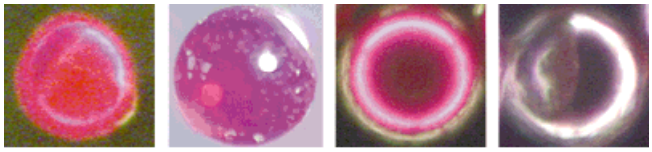

Figure 3. Shown are optical photographs of the four resins in the absence of fluorescence excitation. The ArgoPore and Tentagel beads are broken to show the inside of the bead (the glass and polystyrene beads are shown as whole beads).

Conclusion

In conclusion, the uniform distribution of reactive sites seen in ArgoPore and glass beads demonstrates the effective functionalization process in contrast to that for polystyrene and Tentagel. As a single bead from each of these four solid supports contains approximately the same number of attachment sites, the data show that there is a higher effective concentration at the surface of both polystyrene and Tentagel beads relative to the macroporous resins. The surface congestion of amines seen with polystyrene and Tentagel can be thought of as a functionalized "shell" of amino groups on the bead's outer 5–10 μm. The thickness of the functionalized outer shell seen with polystyrene and Tentagel may depend on the degree of crosslinking in a specific batch of resin and the precise conditions under which the support was prepared and functionalized. Therefore this functionalized shell may be batch specific, causing the resin to have different characteristics from one batch of resin to the next.[25] Optical analysis is a useful method to determine rapidly the quality of the batch and the depth of the functionalized shell, thus setting a standard from which all resin synthesis can be examined. This technique should be useful for future studies on polymerization to evaluate reaction-site distribution, and may lead to predictions in resin reactivity and behavior.

Experimental Section

ArgoPore, glass, polystyrene, and Tentagel were obtained from Argonaut, Schuller, Nova Biochem, and Rapp. Polymere, respectively. In a typical experiment, the fluorescently labeled beads were prepared by coupling 2.5 equivalents of the *o*-nitrobenzyl linker[26] with 4 equivalents of [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) and 8 equivalents of Hunigs base (DIPEA) in 1:1 ratio of DMF:DCM. The reaction vessel was rotated for 18 hours and complete coupling was determined by use of a ninhydrin analysis, whereupon extensive washing (3 × 2 mL with THF, DMF, MeOH, THF, DMF, MeOH, THF, DMF, MeOH, THF) and subsequent deprotection with TFA yielded the desired amino resin. After drying the beads at 50 °C in vacuo, addition of the carboxytetramethyl rhodamine dye (3 equivalents) in DMF and Hunigs base, rotation of the reaction vessel for 18 hours, washing as listed above, and repetition of this procedure three times, resulted in complete coupling of the rhodamine dye to the resin. The final washing procedure to remove excess dye involved the use of 1 × 2 mL of DCM, MeOH, and DCM for 12 hours each, upon which the final wash was clear, with no dye being visually detected.[27] Samples were dried in vacuo at 40 °C for 12 hours. Elemental analysis for the percentage nitrogen of the resins coupled with dye showed high loadings on all resins: ArgoPore 3.11 %N = 0.444 mmol g^{-1} (~90% of theoretical), glass 0.43 %N = 0.0614 mmol g^{-1} (~70% of theoretical), polystyrene 2.38 %N = 0.34 mmol g^{-1} (~80% of theoretical), and Tentagel 1.06 %N = 0.15 mmol g^{-1} (~70% of theoretical).[28]

The samples were prepared by placing approximately 5 mg of fluorescently labeled beads onto a standard microscope slide. A cover slip was placed on top of the beads and glued in place. These beads were then examined on a typical optical analysis microscope with a 20X power lense. The resolution of this method is 0.41 μm in the *x* and *y* direction.

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[18] Beads were coupled three times with three equivalents of dye each time and our experience from using qualitative analysis on similar reactions indicates this is sufficient for complete coupling. Further, the elemental analysis indicated that high loadings of dye were achieved (see Experimental Section) and therefore we conclude all available amino sites were functionalized with rhodamine dye. Experiments using a linker were examined as they were in conjunction with other experiments in our laboratory; however, the linker played no role in the optical analysis.

[19] Multiple beads of each type were examined using optical analysis to ensure that a representative bead for each type was shown. Experiments were done on beads with no photolinker and a direct coupling of Rhodamine dye to the bead; they produced results indistinguishable from those with linkers.

[20] Integration of the photon count yields approximate values in the outer 5 – 10 microns versus photons on the inner 50 – 90 microns of each slice. The values obtained are an average of several beads by using two slices in the approximate center of the bead.

[21] The relative intensity of the beads were not compared with each other as multiple factors contribute to the fluorescence intensity for each bead, including bead size and loading.

[22] This may also be a function of the amino-terminated PEG chains.

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[28] Other reaction sequences and subsequent Fmoc analysis have shown that frequently the highest loading actually achieved after a number of synthetic transformations are similar to the values seen from elemental analysis (i.e., 70 – 80 % for all resins). Thus it appears some amines on the manufactured beads may not be accessible. Further, as these samples were stored for 8 months before the elemental analysis and decomposition may have occurred (see ref. [11d]).

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