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Production, Analysis, and Application of Spatially Resolved Shells in Solid-Phase Polymer Spheres

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Abstract: Two-photon fluorescence microscopy has been used to interrogate the interior functionality of polymer resin beads. By employing this technique, the spatial distribution of the initial functionality contained within the polymer matrix has been determined. Spatially resolved, concentric shells were then produced synthetically in these polymer spheres via a series of protection/deprotection reactions in which two-photon fluorescence microscopy was employed to monitor each successive step. To demonstrate the potential utility of these techniques in combinatorial screening, a set of beads was prepared containing a unique tripeptide sequence in each of the three concentric shells within each individual bead. The set was then screened for the binding affinity of each tripeptide toward a fluorescent ligand.

Introduction

The synthesis of organic molecules on solid-phase synthesis beads has experienced an explosion of interest since Merrifield's pioneering work in the peptide area several decades ago.[1−6] In large part, this renaissance has been driven by the advent of combinatorial chemistry, which takes advantage of the ability to synthesize large and diverse libraries of compounds efficiently on solid support.[7−9] Despite the tremendous practical advantages afforded by solid-phase synthesis, relatively few reports have appeared in which a direct determination of the on-resin chemistry has been made. Examples of techniques that have been used include radiography,[10] nanoprobe nuclear magnetic resonance,[11] single-bead fluorescence microscopy,[12] IR spectroscopy,[13] and optical analysis.[14,15]

The in situ screening of libraries of resin-bound compounds represents a frontier in this area of research. However, the approach has to date been applied with caution, in part because of questions concerning the spatial location of molecules on, or within, a given bead.[16] A related question concerns the diffusivity of macromolecular targets within the polymer matrix that comprises the solid-phase bead.[17−19]

Here we report the direct observation of both on-resin bead functionalization and ligand binding, using a high-resolution, three-dimensional optical technique. Two-photon microscopy has been used to carry out in situ cross-sectional analysis of individual synthesis beads that have been functionalized with peptides that bind other fluorogenic molecules. The ability to observe the properties of the interiors of synthesis beads has further allowed us to develop a verifiable synthetic route to multiple-shelled beads wherein different shells are functionalized with different compounds. We have used this technique to prepare three-shelled beads in which each shell is functionalized with a unique tripeptide sequence. On the basis of the individual molecular recognition properties of each tripeptide sequence, we demonstrate the shell-selective binding of unique ligands within the interiors of the individual beads.

Results and Discussion

Two-Photon Fluorescence Microscope. The technique used to observe the interiors of beads is two-photon laser fluorescence microscopy.[20] The probability for two-photon excitation (TPE) of a molecule is proportional to the square of the intensity of the light used for excitation. As a result, TPE is an efficient process only when and where the excitation light is focused tightly. Since TPE is limited to a tight focal volume, three-dimensional resolution comparable to that of confocal micros-

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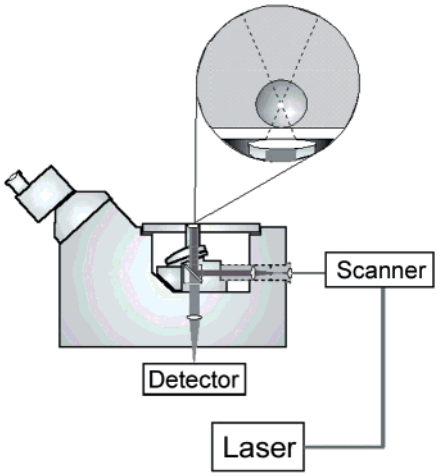

Figure 1. Diagram of the TPM. The inset depicts excitation of a small volume of fluorophores at the focal point of the microscope objective within a polymer sphere. Three-dimensional images are collected by raster scanning the focal point of the objective through the entire sample and detecting the emitted fluorescence at each step of the scanning. A computer program and PC card synchronize the scanning mirrors with the detector and output the image as a function of beam position.

copy can be attained readily.[21,22] However, TPE microscopes generally employ an excitation source in the near-infrared, as opposed to the visible or ultraviolet sources usually used in confocal microscopes. Thus, the interiors of materials that have high optical density in the visible and/or ultraviolet but not in the near-infrared can be imaged readily with two-photon fluorescence microscopy (TPM). It is for this reason that photodamage is significantly reduced as compared with confocal microscopy, in which the out-of-focus fluorophores are also excited. Because the excitation occurs only at the focal point, TPM provides a method for probing small volumes within a larger system. By moving the focal point of the microscope objective to different areas of a sample, information concerning the chemical composition of each of the probed areas can be gathered.

The two-photon microscope shown in Figure 1 has been described in detail previously.[23] The excitation source for TPE is a home-built, argon-ion-pumped Ti:sapphire laser that produces 40 fs pulses with a center wavelength of 800 nm at a repetition rate of 76 MHz. The laser beam is steered by a galvanometer-driven mirror-scanning system that directs the beam through an eyepiece into the rear port of a Zeiss Axiovert 100 inverted epifluorescence microscope. The laser is reflected by a dichroic mirror into a 10× objective that focuses the beam into the sample. Fluorescence from the sample is collected by the same objective, passes through the dichroic mirror, and is then focused onto a single-photon-counting avalanche photodiode. Photon counting is synchronized with the raster-scanning of the excitation spot using a home-built card installed in a personal computer. Typical scans are obtained with an excitation power of 5 mW. The sample is contained in a specially constructed, air- and solvent-tight cell that employs a thin

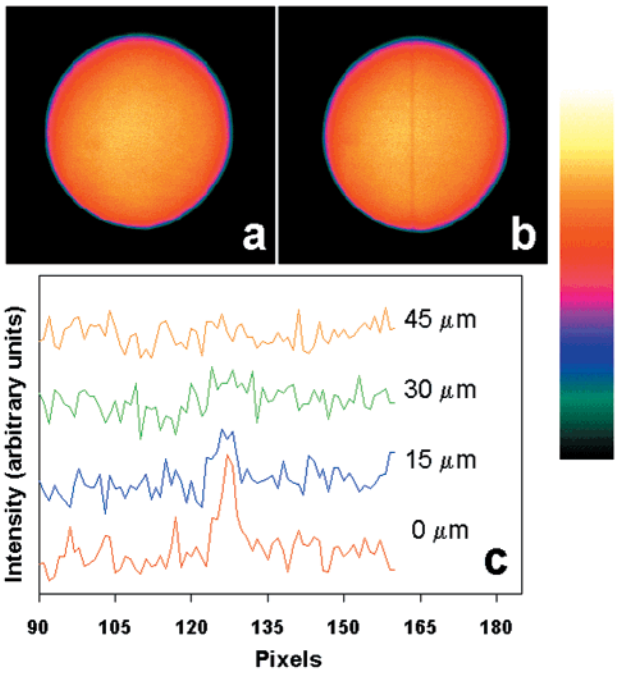

Figure 2. (a) Cross-sectional TPE image of a TentaGel sphere with the dansyl fluorophore bound to the amine functionalization. (b) The same sphere as in (a) except that there is a vertical line photobleached through the center of the cross section. The color of images a and b is determined by the intensity of the fluorescence detected (see scale to the right of the images). (c) The chart shows the difference in intensities between a horizontal line through the sphere before and after photobleaching. The lower trace (red) was taken from the same plane as the photobleaching, the second trace from the bottom (blue) was taken at an image plane that is displaced from the plane of photobleaching by 15 μm, the green trace is displaced by 30 μm, and the orange trace is displaced by 45 μm.

coverslip as the transmission window. The synthesis beads are loaded into the cell along with the solvent. The beads can either be prepared as desired in advance or the appropriate reagents for a given reaction can be added to the sample cell and the reaction dynamics can be observed in situ in real time.

Distribution of Functionality. To assess the uniformity of functionalization throughout the polymer spheres, a set of TentaGel[24] spheres was prepared in which all of the available amines were reacted with a fluorophore. Dansyl chloride was chosen as the fluorescent species for several reasons: (1) it reacts readily with primary amines, (2) it is nonfluorescent before reaction with a primary amine, (3) it has a large, solvent-dependent Stokes' shift that minimizes the effects of reabsorption,[12] and (4) it has a decently large two-photon cross section. Cross-sectional images of the dansylated spheres were collected with the TPM. Figure 2a is a representative sample of the images obtained. The color of the image is determined by the fluorescence intensity detected at each pixel (see color scale at the right of Figure 2). The fluorescence appears uniform throughout the majority of the sphere with a slight decrease near the surface of the sphere. We believe that there are two reasons for this decrease in intensity: (1) a slightly lower density of the polymer at the surface of the sphere and (2) the axial resolution of the microscope is not as good as the in-plane resolution.

After having collected the image seen in Figure 2a, the microscope was programmed to scan a vertical line through the

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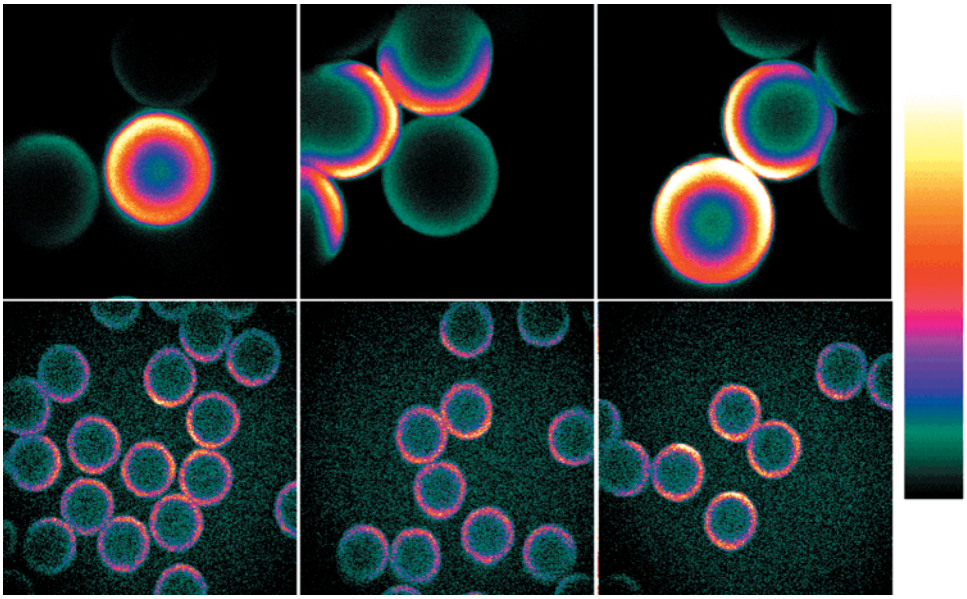

Figure 3. The top three images are 90-μm TentaGel spheres (amine functionalized) that have been treated with a limiting amount of dansyl chloride. The bottom three images are of 30-μm amine-functionalized polystyrene spheres that have been treated with a limiting amount of dansyl chloride. The color of the images corresponds to the intensity detected at each pixel (see scale at right). The maximum intensity value is different for each set (top and bottom).

center of the sphere repeatedly, resulting in the bleaching of a portion of the dansyl fluorophores along the line of scanning (Figure 2b). The intensity trace labeled "0 μm" is the difference in intensity of the 10 horizontal lines that comprise the center of the sphere before photobleaching (Figure 2a) and after photobleaching (Figure 2b). The three other traces in Figure 2c were extracted from cross-sectional images that were collected from planes offset from the bleached plane by the distance that labels each trace. The peak that appears at about the 128th pixel in the "0 μm" trace is a result of the change in fluorescence intensity caused by the photobleaching of some dansyl fluorophores. By translating the focal plane 15 μm away from the bleaching plane, the effects of the bleaching are seen to be less prevalent, and at 30 and 45 μm the effects of photobleaching are no longer discernible. This experiment demonstrates the three-dimensional capabilities of TPM and of the method by which we are able to probe the internal features of the solid supports.

Kinetics. The next experiment performed concerned the kinetics of the reaction between the dansyl chloride and the amine functionalization of the spheres. Since dansyl chloride is not fluorescent until reaction with a primary amine, monitoring the fluorescence emitted from the spheres as a function of time produces information concerning the relative rates of diffusion of the dansyl chloride into the beads and of reaction of the dansyl choride with the beads. However, because TPM can probe the interior of the beads, we were able to monitor the fluorescence emitted from different areas of a single sphere by imaging the cross section of a sphere prior to the addition of a limiting amount of dansyl chloride and at time intervals after the addition of the dansyl chloride. Thus, a set of spheres that had been solvated with DMF was placed in a sample cell containing DMF and diisopropylethylamine (DIPEA). A single sphere was found, and the focus of the microscope was adjusted such that a cross section containing the center of the sphere was in the focal plane of the microscope. A small volume of a dilute solution of dansyl chloride in DMF was added to the sample cell, and images were collected as a function of time. Representative images from six different time series are shown in Figure 3. The top images are of 90-μm TentaGel spheres, while the bottom images are of 30-μm polystyrene spheres. As can be seen in these representative cross sections, all of the spheres imaged contained a fluorescent outer portion (shell), while the inner core remained nonfluorescent. This result implies that the reaction of the dansyl chloride with the bead-bound amines occurs at a higher rate than does the diffusion of the dansyl chloride into the beads. Thus, the resulting beads have two distinct areas of functionalization, the outer shell containing the dansyl fluorophore and the inner core of unreacted amines.

Although all of the beads in a single kinetics experiment contained a fluorescent shell and a nonfluorescent core, the width of the fluorescent shell varied from sphere to sphere. To alleviate this situation and to test the viability of preparing shells under typical reaction conditions, an experiment was devised such that the reactions were carried out in spin tubes that could be agitated by rotation. A set of 10 spin tubes containing 90-μm TentaGel spheres was prepared, along with 10 solutions containing incremental amounts of dansyl chloride. Each of the spin tubes was treated with one of the dansyl chloride solutions, and the reactions were agitated on a spin apparatus. After the allotted reaction time, the solvent in the two spin tubes containing the highest concentrations of dansyl chloride remained slightly colored, while the solvent in the other eight tubes was colorless. Cross-sectional images of samples from each spin tube were collected and analyzed (Figure 4). The intensity traces above each of the cross sections were produced by integrating the 10 horizontal lines that comprise the center of the spheres. It is apparent that the fluorescent shell initiates at the outer surface of the shell and grows inward, culminating in a fully dansylated bead as the concentration of dansyl chloride is increased. Samples 9 and 10, the beads treated with the two highest concentrations of dansyl chloride, were both fully dansylated, thus explaining the yellow color that remained in the solvent after the allotted reaction time. The agitation of the

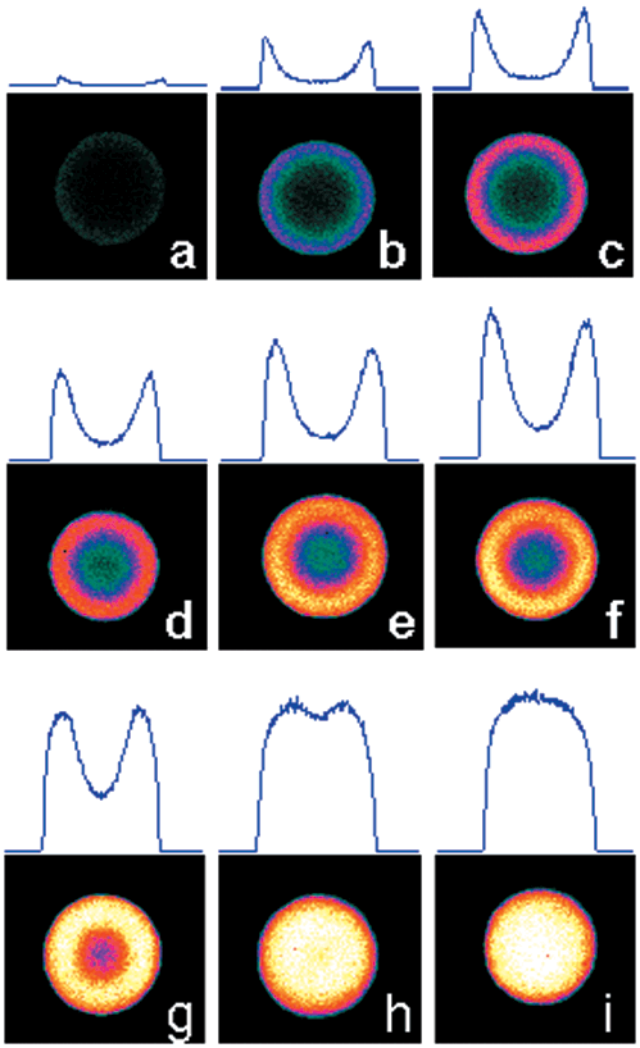

Figure 4. Images of 90-μm TentaGel spheres that have been sequentially treated with successive aliquots of dansyl chloride. The traces above each image are the corresponding intensity values for a horizontal line passing through the center of the sphere. The scan area of each image is identical, and each intensity trace is scaled to the same maximum value. The images are colored according to fluorescence intensity.

reactions also resulted in the fluorescent shell being quite uniform from bead to bead throughout an entire sample.

These results reinforce the idea that the reaction of dansyl chloride with the bead-bound amines occurs at a faster rate than does the diffusion of the dansyl chloride into the beads. The results also bolstered our belief that multiple, spatially resolved shells could be synthesized chemically in the polymer spheres.

Three-Shelled Spheres. On the basis of the results from the kinetic studies, we devised a strategy to produce spheres containing three spatially resolved shells. The goal was to prepare beads that contained a fluorescent outer shell and inner core and a nonfluorescent intermediate shell. Our strategy was based on a series of protection/deprotection reactions using 9-fluorenylmethyl chloroformate (Fmoc-chloride) as the amine-protecting group. Fmoc-chloride was chosen because its reaction kinetics with primary amines are similar to those of dansyl chloride.

The scheme shown in Figure 5 outlines the procedure we used to produce three shells in TentaGel spheres. After each step in the procedure, a small sample was removed from the

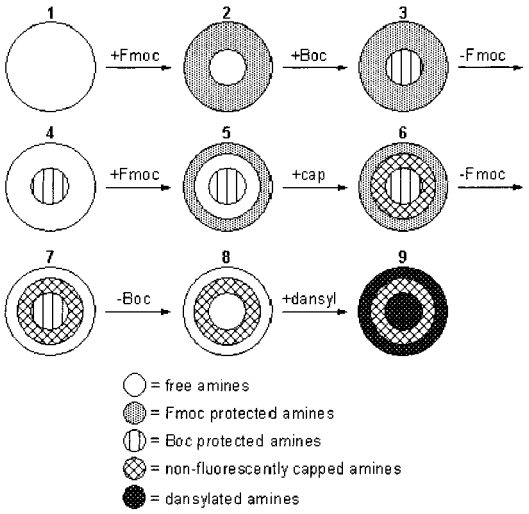

Figure 5. Scheme for the production of three-shelled beads containing the dansyl fluorophore in the outer and inner shells and a nonfluorescent species in the intermediate shell.

spin tube and treated with an excess of dansyl chloride. Thus, when cross-sectional images of these samples are collected, fluorescent areas show the position of the remaining free amines (Figure 6). Initially the 90-μm TentaGel spheres **1** were treated with a limiting amount of Fmoc-chloride **2** (Figure 6a), and the remaining free amines were protected with the *tert*-butoxycarbonyl group (Boc) **3** (Figure 6b). Fmoc and Boc are orthogonal protecting groups − Fmoc is base labile and Boc is acid labile. The Fmoc was cleaved **4** (Figure 6c), and a second treatment of Fmoc-chloride was applied to the beads (Figure 6d) − this treatment contained a lower concentration of Fmoc-chloride than did the first treatment. The free amines were capped **6** (Figure 6e). Both the Fmoc (Figure 6f) and the Boc were cleaved from the beads **8**, and dansyl chloride was reacted with the free amines **9** (Figure 6g). Figures 6a−g are false color images, in which the color is representative of the fluorescence intensity, according to the scale to the right of the figure.

Another set of three-shelled spheres was prepared in a manner similar to that above, except the concentrations of the Fmoc-chloride treatments were reduced. Figure 6h is a true-color, cross-sectional image taken with the CCD camera of a bead from this second batch. Reducing the concentration of the Fmoc-chloride during synthesis resulted in a reduction in volume of the outer two shells and gave a much larger inner core. These results, specifically the volume contained in the inner core of Figure 6h, demonstrate the possibility of synthesizing more than three shells per bead.

Five-Shelled Spheres. To test the effectiveness of our experimental procedure for producing a greater number of shells per sphere, we synthesized 90-μm TentaGel spheres containing five shells. The procedure used to prepare the five-shelled spheres is shown in Figure 7. The same basic principles of protection/deprotection reactions that were followed for the three-shelled spheres were also used for the five-shelled spheres. However, unlike the procedure for the three-shelled spheres in which the shells were produced from the core to the outer shell (inner core produced first, outer shell produced last), for the five-shelled spheres the outer shell was produced initially, while the inner core was produced last. As with the procedure for the three-shelled spheres, Fmoc-chloride was used as the initial

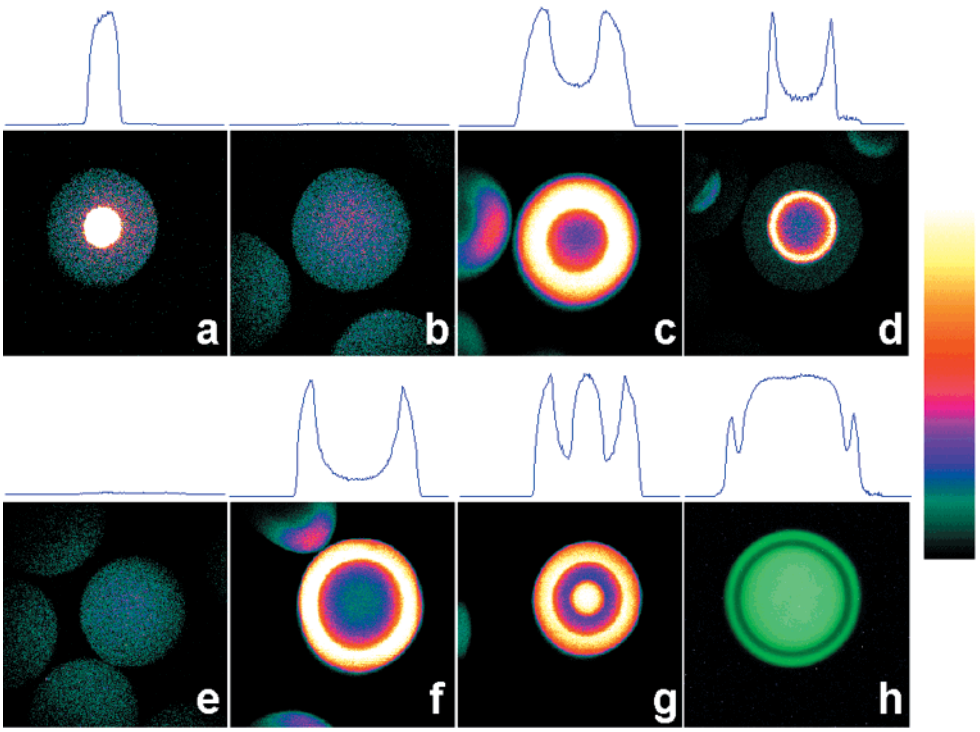

Figure 6. Images a−g are successive steps in the procedure for producing three-shelled spheres; the color of these images arises from the intensity of the fluorescence detected at each pixel (see scale to right of images). Image h is a true-color image of a three-shelled sphere. Notice the difference in the shell sizes between image g and image h; the thickness of the shells is controllable by varying the amount of Fmoc-chloride in each step. The scan area of images a−g is identical, and an intensity trace through the vertical center of each sphere is provided above each image. Image c clearly shows a gradual increase of fluorescence intensity as one moves from the outer surface of the sphere to the core. This rate of change with distance into the sphere is highly dependent on the solvent employed. Solvents in which the polymer is well solvated cause the spheres to swell to a greater volume than those that do not solvate the polymer as well. The rate of change of the intensity is more gradual for the solvents in which the spheres swell to a greater extent, while the solvents that do not solvate the polymer as well provide a steeper intensity curve. Because the polymer at the outer edge of the sphere has greater degrees of freedom than the polymer in the core of the sphere, we believe that solvation affects the polymer at the surface of the sphere to a greater extent than the polymer near the core of the sphere. A reduction in image resolution is also caused by the depth of the focal volume. The bleaching experiment in Figure 2 showed that there was some photobleaching damage at a plane 15 μm from the desired image plane. Thus, some amount of out-of-focus fluorescence reduces the resolution of the image.

amine-protecting group, while 2-acetyldimedone (Dde) was used as the secondary protecting group instead of Boc. Fmoc and Dde are not completely orthogonal protecting groups − Dde is stable to the basic solution used to remove the Fmoc, while Fmoc is removed by the conditions necessary to cleave Dde. As can be seen in Figure 7, at the steps in the synthesis of the shells when Dde was cleaved, no Fmoc was present on the beads, thus the semiorthogonal relationship between Fmoc and Dde did not affect the synthesis of the shells. Representative cross-sectional images of the five-shelled spheres are shown in Figure 8. These are true-color images that were collected in a series of solvents to demonstrate the solvent effect on the Stokes' shift of the dansyl fluorophore.

While we were able to produce the beads shown in Figure 8 by means of the method in Figure 7, production of the shells from the outer shell working inward proved to be fairly difficult. As the synthesis of the shells progresses, the production of each successive shell becomes more difficult. The primary obstacle is the reduction in the volume of each shell. If we assume that the thickness of each shell is constant, we can produce five 9-μm thick shells in each 90-μm bead. The difficultly lies in the fact that the volume of the outer shell is over 60 times greater than that of the inner core. Thus, preparing solutions containing the correct amount of Fmoc-chloride proved to be quite tedious. However, each step of the synthesis was monitored with two-

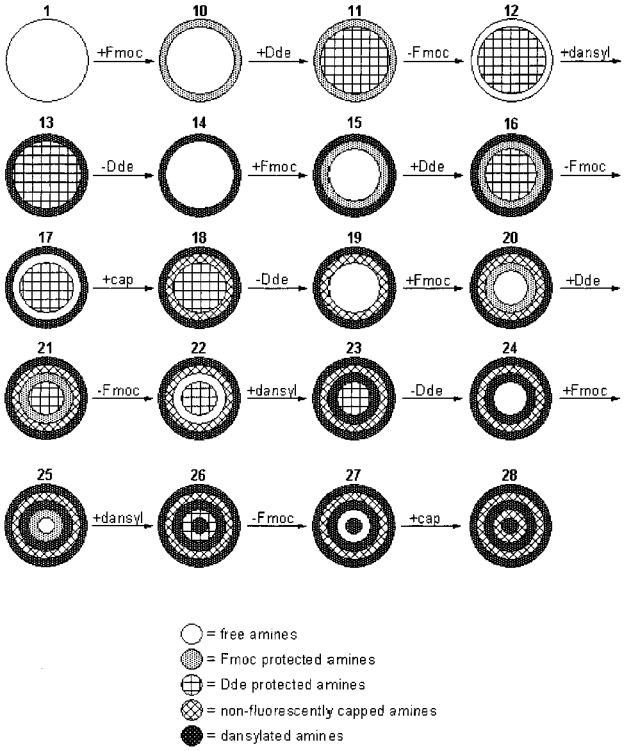

Figure 7. Representative scheme for the production of five-shelled spheres containing alternating fluorescent and nonfluorescent shells.

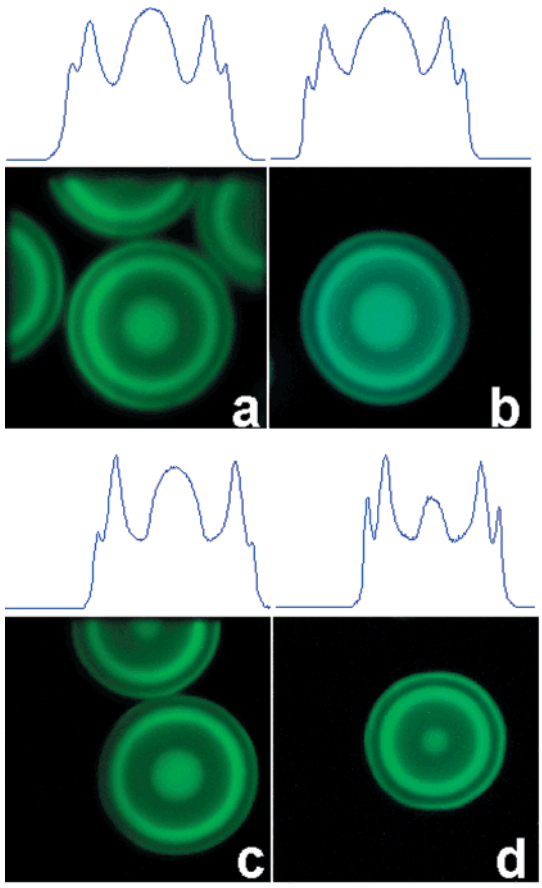

Figure 8. Images of five-shelled spheres having the dansyl group as the fluorophore. All four images are in true color and were taken with the CCD camera. The images were obtained in (a) DMF, (b) toluene, (c) acetonitrile, and (d) dioxane. The scan area of each image is identical, and intensity traces extracted from the vertical center of the spheres appear above each image. Note that the resolution is similar in each solvent, but that there is a color change due to the solvatochromism of the fluorophore.

photon microscopy, and, if the shell was not the desired thickness, the Fmoc was cleaved, and the reaction was repeated.

Three-Shelled, Three-Dye Spheres. The next objective was to determine the degree of cross-talk between the individual shells. To accomplish this task, we prepared three-shelled spheres that contained a unique fluorophore in each shell. The fluorophores were chosen such that each emitted fluorescence that was significantly different in wavelength from that of the other two fluorophores. The three fluorophores chosen were dansyl chloride (green fluorescence), Rhodamine B (orange fluorescence), and Coumarin 343 (blue fluorescence). Dansyl chloride reacts with the amine functionalization of the beads through a sulfonyl chloride group, while Rhodamine B and Coumarin 343 both react through a carboxylic acid group.

Since each of the three dyes possesses a significantly different fluorescence quantum yield, experiments were run to determine the necessary loading of each dye such that each shell would produce a fluorescence intensity similar to the other two shells. Sets of spheres were prepared that were fully functionalized with one of the fluorophores, and images of each were taken with the microscope. The dansyl fluorophore produced the least fluorescence, while the Coumarin 343 emitted the most fluorescence. Thus, several dilutions of Rhodamine B and Coumarin 343 were prepared using benzoic acid as the competitive reagent. Each of the successive dilutions was reacted with the polymer

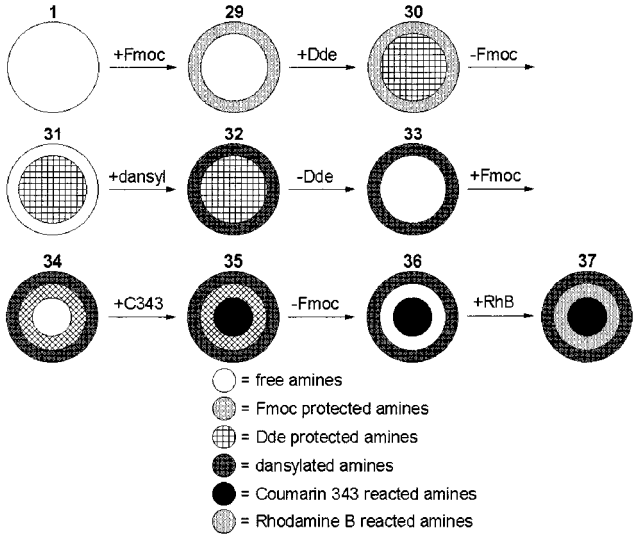

Figure 9. Scheme for the production of three-shelled beads containing individual fluorophores in each shell.

spheres, and images were collected to determine the dilution that produced a fluorescence intensity similar to that of the dansylated beads. Beads reacted with a 1:5 (Rhodamine B:benzoic acid) dilution or a 1:100 (Coumarin 343:benzoic acid) dilution were found to produce fluorescence intensities similar to that of the dansylated spheres.

Figure 9 shows the sequence of reactions that was used to prepare the three-dye beads. The procedure is similar to that of the five-shelled spheres, in which the outer shell was produced first followed by the inner core and intermediate shells. Fmoc and Dde were again employed as the primary and secondary protecting groups. After completion of the synthesis, **37** was rinsed three times with MeOH, three times with 5 mM sulfuric acid in MeOH, and three times with acetonitrile. The beads were allowed to equilibrate for 2 h in acetonitrile. Figure 10b is a true-color, cross-sectional image of one of these beads collected with the CCD camera. To collect spectra of each shell, a transmission grating was inserted in the fluorescence path just before the CCD camera. The microscope was programmed to scan a vertical line through the center of the bead, and the CCD camera was set to intensity mode (total intensity was collected without color information). Figure 10a shows the result of a scan of this sort. The vertical line seen at the left of the figure is the zero-order diffraction line, while the first-order diffraction (the spectra) is seen on the right of the image. Figure 10c shows the spectra of the shells of the bead with the corresponding wavelengths − the color for this image is determined by the amount of intensity detected, and the scale is to the right of the image. The wavelength scale was determined by imaging the spectra of several solutions of dyes with known fluorescence maxima. The spectra clearly show the spectral transition from one shell to the adjacent shell and demonstrate that there is relatively little chemical cross-talk between the different shells.

Three-Shelled, Three-Tripeptide Spheres. In 1994, Wennemers and Still demonstrated that certain tripeptide sequences bound to poly(ethylene glycol)-polystyrene polymer resins bound simple dye molecules selectively in water.[25] Using a similar approach, here we demonstrate the ability to screen the

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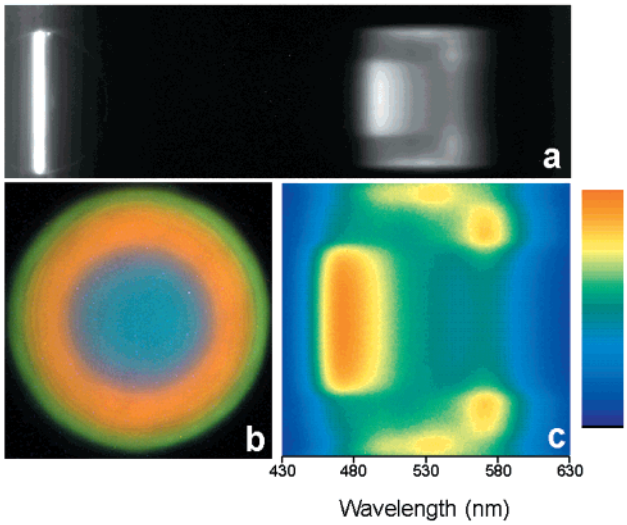
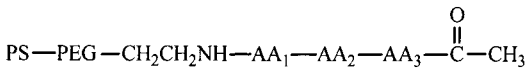


Figure 10. Image a is the diffraction pattern of the fluorescence emitted when the excitation source is allowed to scan a vertical line through the center of the sphere. The zero-order diffraction is the vertical line on the left side of image a, while the first-order diffraction is to the right. Image b is a true-color image of a 90-μm TentaGel sphere in which three shells have been synthesized. The outer shell has been modified with dansyl chloride, the second shell with Rhodamine B, and the core with Coumarin 343. Image c is the first-order diffraction pattern taken from image a with wavelengths assigned. Notice the clear separation of each shell of the sphere in the diffraction pattern.

binding of dye molecules to three unique, spatially addressable peptide sequences on a single resin bead. While Wennemers and Still employed colorimetric means to assay the binding of the dyes to the bead-bound tripeptides, we used a fluorogenic screening method. Of the 10 dyes that Wennemers and Still tested for selective binding, Rhodamine B was the best suited for fluorescence analysis with our system. Thus, we chose three tripeptide sequences − one that binds Rhodamine B strongly and two that bind the dye weakly. The binding tripeptide was an all-asparagine (NNN) sequence with the reactive side chain amines trityl protected. The nonbinding sequences were the all-valine (VVV) and the all-leucine (LLL) tripeptides. The terminal amine of all three sequences was capped with an acetyl group, yielding sequences with the general structure:



As a control experiment, a set of beads was prepared in which the all-asparagine tripeptide was synthesized throughout each entire bead. A sample of these spheres was washed several times with water and then immersed for 12 h in a dilute solution of Rhodamine B in water. The dilute Rhodamine B solution was slightly pink in color prior to the addition of the all-asparagine beads. After 12 h, the solution was colorless, while the beads were pink in color. Cross-sectional images of these spheres were collected, and a sample is shown in Figure 11. Fluorescence from the Rhodamine B can be seen evenly throughout the entire sphere. One concern in imaging the interiors of beads using TPM was that the difference in the indices of refraction between the water and the TentaGel beads would cause deflection of the excitation beam at the surface of the solid supports, yielding images that were not representative of the actual characteristics of the beads. Thus, a sample of the three-shelled spheres

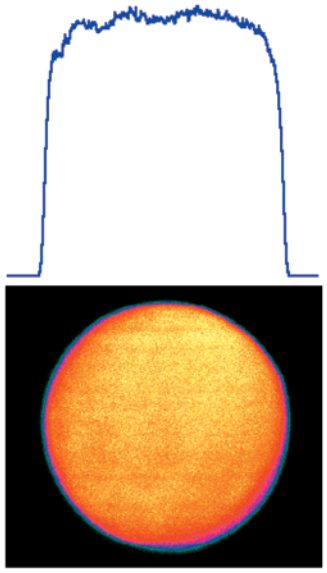


Figure 11. A cross-sectional TPE image of an all-asparagine bead in a dilute aqueous solution of Rhodamine B. The trace above the image is the fluorescence intensity through the vertical center of the image.

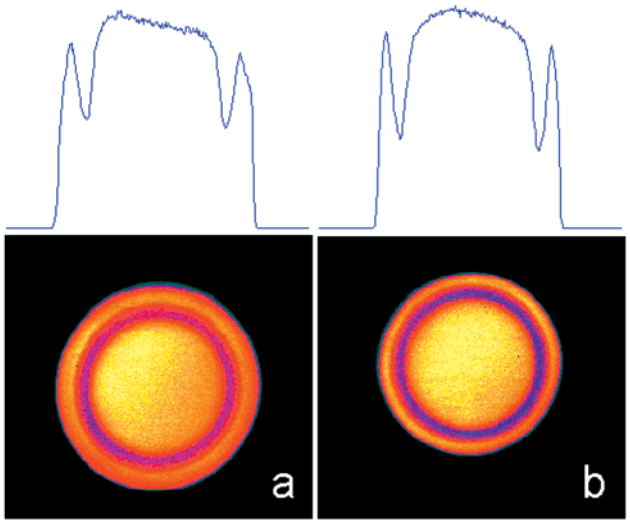


Figure 12. Cross-sectional TPE images of the dansyl/cap/dansyl beads. Image a was taken in DMF, and image b was taken in water. The scan area of each image is identical, and intensity traces through the vertical center of each sphere are shown above each respective image. The difference in the swelling properties of the two solvents is apparent. The polymer is well solvated by DMF, while water does not solvate the polymer as well. As noted previously, the effect of solvation has a direct effect on the resolution of the outer shell.

containing the dansyl fluorophore in the outer and inner shells was imaged in DMF (Figure 12a) and in water (Figure 12b). Although the bead volume is smaller in water than in DMF due to the swelling characteristics of the TentaGel, both images show three clearly resolved shells. We can therefore conclude that the difference in the indices of refraction is not affecting the image quality to a significant extent.

Three different sets of tripeptide beads were prepared with the positions of each tripeptide sequence varied from set to set (Table 1). The sequence for the synthesis of sample A from Table 1 is shown in Figure 13. The procedure is effectively the three-dye bead synthesis with the dye coupling reactions replaced with tripeptide syntheses. The syntheses of samples B and C follow that shown in Figure 13 with only the positions

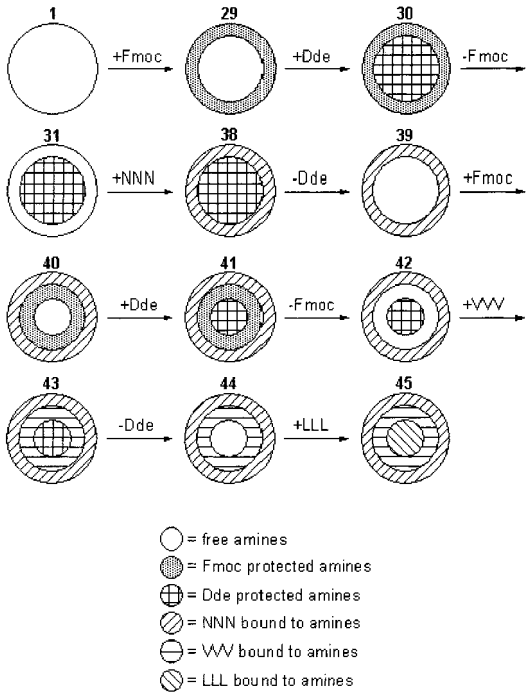


Figure 13. Representative scheme for the preparation of multilayered solid supports. The synthetic procedure for creating sample A is shown here.

Table 1. Positions of the Three-Tripeptide Sequences in Each Shell of Three Different Sample Sets of Beads

	sample		
position	A	B	C
outer	NNN	LLL	VVV
intermediate	VVV	NNN	LLL
inner	LLL	VVV	NNN

of the tripeptide sequences varied. Upon completion of the syntheses, a sample of each different set was collected and washed several times with water, after which the samples were allowed to equilibrate in a dilute solution of Rhodamine B in water for 24 h. Cross-sectional images were obtained for each of the samples (Figure 14). As expected, the all-asparagine tripeptide shell was the only shell to show significant fluorescence, regardless of its position in the bead. These results demonstrate that it is possible to prepare solid supports containing spatially addressable shells in which unique compounds can be synthesized and screened for binding with a verifiable observation technique.

Conclusion

We have described a simple chemical method for synthesizing spatially resolved shells in solid supports. The method is based on the fact that the diffusion rate of solution-phase reactants into the spheres occurs on a time scale that is slower than the reaction of the solution-phase species with the functionality of the solid supports. Thus, by employing Fmoc-chloride, shells can be produced by means of protection and deprotection of the primary amines that are the functional basis of the solid supports. Using this method, we have shown that it is possible to prepare multishelled spheres, each containing unique chemical identities. TPM has been used to monitor each step in the production of the shells. We were also able to construct spheres containing three shells, each modified with a unique tripeptide sequence. Using a fluorescence assay performed with TPM, we were able to screen the peptides in the separate shells simultaneously for binding affinity to Rhodamine B. The ability to prepare and observe the spatial characteristics of multishelled synthesis beads with the assistance of TPM may enable to a number of new techniques in combinatorial chemistry, including potential on-resin screening of single-bead/multiple compound libraries with high-resolution, real-time observation of bead chemistry. Also, the diffusive characteristics of fluorescent species into any porous, nonabsorbing media can be determined with TPM.

Experimental Section

General. All polymer spheres used for this publication contained inherent amine functionality and were purchased from Rapp Polymere. The reagents for the reactions were purchased from Aldrich (unless otherwise noted) and used as delivered without additional purification. Fmoc-protected amino acids were purchased from Advanced Chemtech and 2-acetyldimedone was purchased from Novabiochem; all were used without further purification. Sure-seal DMF was used as the solvent for all reactions. All solutions were prepared just prior to use. The reaction vessels employed for the synthesis reactions were either Whatman filter tubes (3 mL capacity, 5.0 μm pore) or Biorad spin columns (1.2 mL capacity). Unless otherwise noted, rinses were performed with reagent grade solvents using the following protocol: 3 × DMF, 3 × MeOH, 3 × DCM, 3 × DMF, 3 × DCM, 3 × MeOH, and 3 × DMF. Agitation was provided by a rotating apparatus to which the spin tubes were clamped. All reactants are listed in each section, and no additional reactants or coupling reagents were employed. Information concerning the amino acid coupling reactions and the amine-protecting and deprotecting reactions can be found in the reference.[26]

Dansylated Spheres. To a reaction vessel was added 100 mg of TentaGel spheres (90 μm, 0.27 mmol/g NH_2 functionality) and 2 mL of DMF. The system was agitated for 1 h to solvate the beads. The DMF was removed via vacuum filtration, and a solution containing dansyl chloride (36 mg, 0.14 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF was introduced to the beads. The reaction was agitated for 30 min, after which the spheres were rinsed.

Three-Shelled Spheres. To a reaction vessel was added 100 mg of TentaGel spheres **1** (90 μm, 0.27 mmol/g NH_2 functionality) and 2 mL of DMF. The system was agitated for 1 h to solvate the beads. The DMF was removed via vacuum filtration, and the beads were allowed to equilibrate in a solution containing DIPEA (25 μL, 0.14 mmol) in 0.5 mL of DMF for 15 min. A 2 mL aliquot of a 9.5 mM solution of Fmoc-chloride in DMF was added rapidly to the reaction vessel, and the mixture was agitated for 1 h. The beads **2** were rinsed, and a solution of di-*tert*-butyl dicarbonate (100 μL, 0.43 mmol) in 2 mL of DMF was added to the reaction vessel to Boc-protect the remaining free amines. The reaction was agitated for 2 h, after which the beads **3** were rinsed. The Fmoc was cleaved via two 30 min treatments with 20% piperidine in DMF, after which the beads **4** were rinsed. The beads **4** were equilibrated for 15 min in a solution of DIPEA (25 μL, 0.14 mmol) in 0.5 mL of DMF. A 2 mL aliquot of a 4.7 mM solution of Fmoc-chloride in DMF was added rapidly to the reaction vessel, and the reaction was agitated for 1 h. After rinsing, the beads **5** were treated with 2 mL of a 3:1:1 (DMF:acetic anhydride:TEA) mixture and agitated for 90 min. The beads **6** were rinsed and subjected to two 30 min treatments of 20% piperidine in DMF. The beads **7** were rinsed, after which the beads were washed several times with dichloromethane (DCM). A 1:1 mixture of trifluoroacetic acid (TFA) and DCM was added to the reaction vessel, and the system was agitated for 30 min, after which the beads **8** were rinsed. A solution containing

(26) *Novabiochem Catalog*; Calbiochem-Novabiochem Corp.: San Diego, 2000.

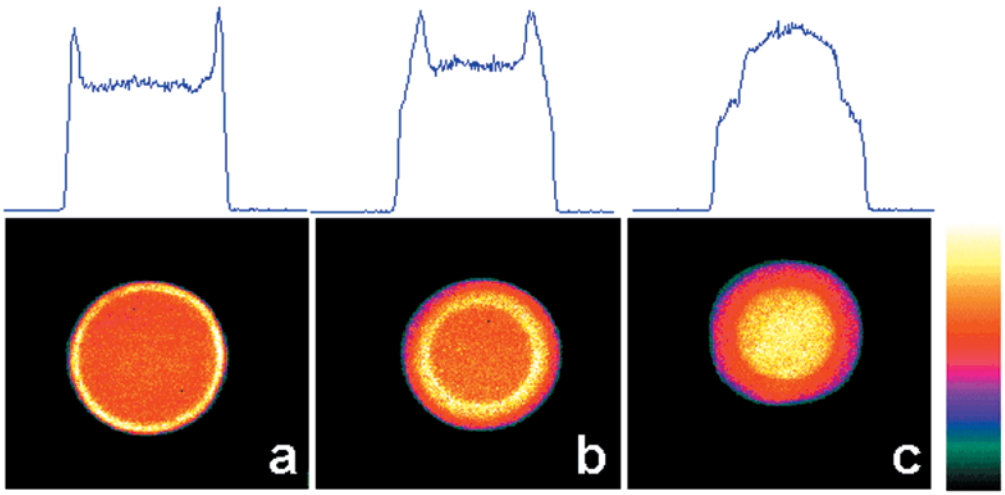


Figure 14. Cross-sectional TPE images of beads of the three-shelled tripeptide beads after equilibrating in a dilute aqueous RhB solution for 24 h. The scan area for each image is identical. The trace above each image corresponds to the fluorescence intensity through the vertical center of the sphere in each image.

dansyl chloride (38 mg, 0.14 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF was added to the beads **8**, and the reaction was agitated for 30 min, after which the beads **9** were rinsed. Cross-sectional images of the beads were collected using TPM (Figure 4).

To obtain the images seen in Figure 4, a small sample of beads was collected after each successive step of the synthesis and treated with a solution of dansyl chloride (38 mg, 0.14 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF. The beads were rinsed and imaged using TPM.

Five-Shelled Spheres. To a reaction vessel was added 100 mg of TentaGel spheres **1** (90 μm, 0.27 mmol/g NH_2 functionality) and 2 mL of DMF. The mixture was agitated for 1 h to ensure complete solvation of the beads in the DMF. The DMF was removed via vacuum filtration, and a solution containing DIPEA (25 μL, 0.14 mmol) in 1 mL of DMF was added to the reaction vessel and agitated for 15 min. A 1 mL aliquot of an 8.1 mM solution of Fmoc-chloride in DMF was added rapidly to the filter tube, and the reaction was agitated for 1 h. The resulting beads **10** were rinsed, a solution containing 2-acetyldimedone (50 mg, 0.27 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF was added to the beads, and the reaction was agitated for 90 min. After rinsing, the beads **11** were subjected to three 5 min washes with 20% piperidine in DMF. The beads were washed with three aliquots of DMF between each piperidine treatment and were rinsed after the third treatment. A solution of dansyl chloride (38 mg, 0.14 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF was added to the beads **12**, and the reaction was agitated for 30 min, after which the beads **13** were rinsed. The beads **13** were subjected to three 5 min washes of 2% hydrazine in DMF with DMF rinses between each hydrazine wash and a complete rinse after the final hydrazine treatment. The beads **14** were equilibrated in a solution containing DIPEA (25 μL, 0.14 mmol) in 1 mL of DMF for 15 min. To the reaction vessel was added a 1 mL aliquot of a 5.4 mM solution of Fmoc-chloride in DMF. The reaction was agitated for 1 h, after which the beads **15** were rinsed. A solution containing 2-acetyldimedone (50 mg, 0.27 mmol) and DIPEA (2 μL, 0.14 mmol) in 2 mL of DMF was added to the beads **15** and agitated for 90 min. The beads **16** were rinsed and treated with three 5 min washes of 20% piperidine in DMF with DMF rinses between the piperidine treatments and a thorough rinse after the third piperidine treatment. A 2 mL aliquot of a 3:1:1 (DMF:acetic anhydride:TEA) mixture was added to the beads **17** and agitated for 90 min, after which the beads **18** were rinsed. The beads **18** were treated with three 5 min washes of 2% hydrazine in DMF with DMF washes between each hydrazine treatment. The beads **19** were rinsed and allowed to equilibrate for 15 min in a solution containing DIPEA (25 μL, 0.14 mmol) in 1 mL of DMF. A 1 mL aliquot of a 4.1 mM solution of Fmoc-chloride in DMF was added rapidly to the reaction vessel, and the reaction was agitated for 1 h, after which the beads **20** were rinsed. A solution containing 2-acetyldimedone (50 mg, 0.27 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF was added to the beads **20** and agitated for 90 min. The beads **21** were rinsed and subjected to three 5 min washes of 20% piperidine in DMF with a DMF rinse between each piperidine treatment and a thorough rinse after the final piperidine treatment. A solution containing dansyl chloride (38 mg, 0.14 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF was added to the beads **22**, and the reaction was agitated for 30 min. The beads **23** were rinsed and treated with three 5 min washes of 2% hydrazine in DMF with DMF rinses between each hydrazine treatment and a thorough rinse after the final hydrazine treatment. The beads **24** were equilibrated for 15 min in a solution of DIPEA (25 μL, 0.14 mmol) in 1 mL of DMF, after which a 1 mL aliquot of a 2.0 mM solution of Fmoc-chloride in DMF was added rapidly to the reaction vessel. The reaction was agitated for 1 h, and the beads **25** were rinsed. A solution containing dansyl chloride (38 mg, 0.14 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF was added to the beads **25**, and the system was agitated for 30 min, after which the beads **26** were rinsed. The beads **26** were treated with three 5 min washes of 20% piperidine in DMF with DMF rinses between each piperidine treatment and a thorough rinse after the final piperidine treatment. A 2 mL aliquot of a 3:1:1 (DMF:acetic anhydride:TEA) mixture was added to the beads **27**, and the reaction was agitated for 90 min, after which the beads **28** were rinsed.

Each successive step can be monitored by treating a small sample of the beads with an excess of dansyl chloride and by collecting cross-sectional images of the resulting beads.

Three-Shelled, Three-Dye Spheres. To a reaction vessel was added 100 mg of TentaGel spheres **1** (90 μm, 0.27 mmol/g NH_2 functionality) and 2 mL of DMF. The mixture was agitated for 1 h to ensure complete solvation of the beads in the DMF. The DMF was removed via vacuum filtration, a solution containing DIPEA (25 μL, 0.14 mmol) in 1 mL of DMF was added to the beads, and the system was allowed to equilibrate for 15 min. A 1 mL aliquot of a 8.1 mM solution of Fmoc-chloride in DMF was added to the reaction vessel, and the reaction was agitated for 1 h. The beads **29** were rinsed, a solution containing 2-acetyldimedone (50 mg, 0.27 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF was added to the beads, and the reaction was agitated for 90 min, after which the beads **30** were rinsed. The beads **30** were treated with three 5 min washes of 20% piperidine in DMF with DMF rinses between each piperidine treatment and a thorough rinse after the final piperidine treatment. A solution containing dansyl chloride (36 mg, 0.14 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF

was added to the beads **31**, and the reaction was agitated for 30 min. After rinsing, the beads **32** were treated with three 5 min washes of 2% hydrazine in DMF with a DMF rinse between each wash and a thorough rinse after the third hydrazine wash. The beads **32** were allowed to equilibrate for 15 min in a solution containing DIPEA (25 μL, 0.14 mmol) and 1 mL of DMF, after which a 1 mL aliquot of a 15 mM solution of Fmoc-chloride in DMF was added rapidly to the reacton vessel, and the reaction was agitated for 1 h. The beads **34** were rinsed. A solution containing Coumarin 343 (37 mg, 0.13 mmol) dissolved in 5 mL of DMF and a solution containing benzoic acid (318 mg, 2.60 mmol) dissolved in 50 mL of DMF were prepared. Using these solutions, a 1:100 (Coumarin 343:benzoic acid) solution was prepared by transferring 0.5 mL of the Coumarin solution to the benzoic acid solution. A 2 mL aliquot of the 1:100 (Coumarin 343:benzoic acid) solution was transferred to the beads **34**, and HOBt (16 mg, 0.12 mmol) was added. The system was allowed to equilibrate for 30 min, after which DIC (20 μL, 0.14 mmol) was added, and the reaction was agitated for 90 min. The beads **35** were rinsed and subjected to three 5 min washes of 20% piperidine in DMF with DMF rinses between each piperidine wash and a thorough rinse after the final piperidine wash. A solution containing Rhodamine B (62.3 mg, 0.13 mmol) dissolved in 5 mL of DMF and a solution containing benzoic acid (318 mg, 2.60 mmol) dissolved in 50 mL of DMF were prepared. These solutions were combined at a 2:5 (Rhodamine B:benzoic acid) ratio to produce a final molar ratio of 1:5 (Rhodamine B:benzoic acid). A 2 mL aliquot of this solution and HOBt (16 mg, 0.12) was added to the beads **36**, and the system was allowed to equilibrate for 30 min, after which DIC (20 μL, 0.14 mmol) was added. The reaction was agitated for 90 min, after which the beads **37** were rinsed.

All-Asparigine Spheres. To a reaction vessel was added 100 mg of TentaGel spheres **1** (90 μm, 0.27 mmol/g NH_2 functionality) and 2 mL of DMF. The mixture was agitated for 1 h to ensure complete solvation of the beads in the DMF. The DMF was removed via vacuum filtration, and a solution containing Fmoc-Asn(Trt)-OH (80 mg, 0.13 mmol), HOBt (23 mg, 0.13 mmol), and DIC (21 μL, 0.13 mmol) in 2 mL of DMF was added to the beads. The reaction was agitated for 90 min, after which the beads were rinsed. The beads were subjected to three 5 min washes of 20% piperidine in DMF with DMF rinses between each piperdine wash and a thorough rinse after the final piperidine wash. The reaction with Fmoc-Asn(Trt) and washes with piperidine were repeated twice to produce the Asn-Asn-Asn tripeptide sequence. After the final piperidine wash, the terminal amine was capped by agitating the beads for 90 min in a 3:1:1 (DMF:acetic anhydride:TEA) solution. Upon completion of the capping reaction, the beads were rinsed.

Three-Shelled, Three-Tripeptide Spheres. The three samples of the three-shelled beads containing the three unique tripeptide sequences in the various shells of the beads were all produced in similar fashion. The sequence detailing the synthesis of one of the samples (sample A) is detailed in Figure 13. The only difference in the samples is the position of the three-tripeptide sequences. Thus, only the synthesis of sample A will be described.

To a reaction vessel was added 100 mg of TentaGel spheres **1** (90 μm, 0.27 mmol/g NH_2 functionality) and 2 mL of DMF. The mixture was agitated for 1 h to ensure complete solvation of the beads in the DMF. The DMF was removed via vacuum filtration, and 1 mL of DMF and 25 μL of DIPEA were added to the reaction vessel and agitated for 15 min. A 1 mL aliquot of an 8.1 mM solution of Fmoc-chloride in DMF was added rapidly to the reaction vessel, and the reaction was agitated for 1 h. The beads **29** were rinsed, and a solution containing 2-acetyldimedone (50 mg, 0.27 mmol) and DIPEA (25 μL, 0.14 mmol) in 2 mL of DMF was added to the beads, and the reaction was agitated for 90 min, after which the beads **30** were rinsed. The beads **30** were treated with three 5 min washes of 20% piperidine in DMF with DMF rinses between each piperidine treatment and a thorough rinse after the final piperidine treatment. A solution containing Fmoc-Asn(Trt)-OH (80 mg, 0.13 mmol), HOBt (23 mg, 0.13 mmol), and DIC (21 μL, 0.13 mmol) in 2 mL of DMF was added to the beads **31**, and the reaction was agitated for 90 min. The beads were rinsed and subjected to three 5 min washes in 20% piperidine in DMF with DMF washes between each piperidine wash and a thorough rinse after the final piperidine wash. The Fmoc-Asn(Trt)-OH reaction and piperidine washes were repeated twice to synthesize the Asn-Asn-Asn sequence. After the final piperidine wash, the beads were agitated in a 3:1:1 (DMF:acetic anhydride:TEA) mixture for 90 min, after which the beads **38** were rinsed. The beads **38** were treated with three 5 min washes of 2% hydrazine in DMF with DMF rinses between each hydrazine wash and a thorough rinse after the third hydrazine treatment. A solution containing DIPEA (25 μL) in 1 mL of DMF was added to the beads **39**, and the system was allowed to equilibrate for 15 min. A 1 mL aliquot of a 15 mM solution of Fmoc-chloride in DMF was added rapidly to the reaction vessel, and the reaction was agitated for 1 h, after which the beads **40** were rinsed. A solution containing 50 mg of 2-acetyldimedone and 25 μL of DIPEA in 2 mL of DMF was added to the beads **40**, and the reaction was agitated for 90 min, after which the beads **41** were rinsed. The beads **41** were treated with three 5 min washes of 20% piperidine in DMF with DMF rinses between each piperidine treatment and a thorough rinse after the final piperidine treatment. A solution containing Fmoc-Val-OH (46 mg, 0.13 mmol), HOBt (23 mg, 0.13 mmol), and DIC (21 μL, 0.13 mmol) in 2 mL of DMF was added to the beads **42**, and the reaction was agitated for 90 min. The beads were rinsed and subjected to three 5 min washes of 20% piperidine in DMF with DMF rinses between each piperidine wash and a thorough rinse after the final piperidine treatment. The Fmoc-Val-OH reaction and piperdine washes were repeated twice to produce the Val-Val-Val tripeptide sequence. After the final piperidine treatment, the beads were treated with a 3:1:1 (DMF:acetic anhydride:TEA) mixture for 90 min, after which the beads **43** were rinsed. The beads **43** were subjected to three 5 min washes of 2% hydrazine in DMF with DMF rinses between each hydrazine wash and a thorough rinse after the final hydrazine treatment. A solution containing Fmoc-Leu-OH (48 mg, 0.13 mmol), HOBt (23 mg, 0.13 mmol), and DIC (21 μL, 0.13 mmol) in 2 mL of DMF was added to the beads **44**, and the reaction was agitated for 90 min. The beads were rinsed and subjected to three 5 min washes of 20% piperidine in DMF with DMF rinses between each piperidine wash and a thorough rinse after the third piperdine treatment. The Fmoc-Leu-OH reaction and the piperidine washes were repeated twice to produce the Leu-Leu-Leu tripeptide sequence. After the final piperidine treatment, the beads were treated with a 3:1:1 (DMF:acetic anhydride:TEA) mixture for 90 min, after which the beads **45** were rinsed.

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