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Microautoradiographic Identification of Receptor-Ligand Interactions in Bead-Supported Combinatorial Libraries

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Abstract: We have developed a method for the microautoradiographic screening of bead-supported combinatorial libraries with radiolabeled receptor molecules which allows for the identification of individual radioactive beads under a low-power light microscope. A ^{14}C-labeled macrobicyclic receptor was assayed against an encoded combinatorial tripeptide library to establish the efficacy of the technique. The described microradiographic scheme promises to be advantageous in cases where the receptors show only modest discrimination between the members of the screened library or in cases where the individual beads provide only very low radioactivity levels after the equilibration with the radiolabeled receptor.

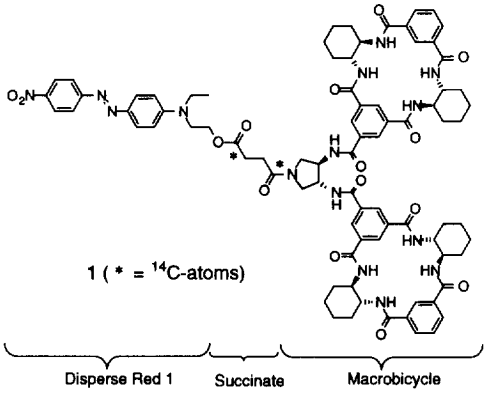
The development of encoded combinatorial chemistry[1-5] created the need for new high-throughput screening technologies.[6-8] We have had success screening "on bead" libraries with substrates coupled to dyes, when these assays have been conducted in organic phase.[9-13] However, we have had difficulties applying this straightforward protocol in aqueous phase. Although the labeling of proteins with hydrophilic dyes is a commonly used protocol for biochemical studies, members of the libraries binding the dye instead of small synthetic receptors have predominated in our assays, thus interfering with efficient screening.[14]

The incorporation of radioisotopes is an elegant approach to label molecules without altering their chemical properties, and radiographic methods find widespread applications in microscopy, medicine, and the studies of intermolecular interactions in biochemical systems. Radiolabeled peptides on solid supports have been used for the sensitive measurements of the kinetics of peptide hydrolysis on solid supports,[15] and recent reports describe the screening of bead-supported combinatorial libraries for intermolecular interactions with ^{125}I-labeled antibodies[16] or for kinase substrates using ^{32}P-ATP as phosphate source.[17] In those experiments the radiolabeled beads are fixed in agarose gels and located by overlaying the gel with a radiographic film. Beads around loci of radiographic signals are recovered and respread at higher dilution. Usually individual beads can be identified after two screening cycles. Although promising, the described experiments showed two drawbacks to us: (A) None of the molecules of our interest contains iodine or phosphorus atoms, and their derivatization with those isotopes bears the potential of effecting their properties. (B) The cycles of recovering and respreading beads seem feasible only in cases where receptors show strong discrimination between the members of the libraries (to allow for the identification of separated spots of radioactivity) and have a sufficiently high affinity to their ligands (to avoid the elution of the receptor while recovering the beads).

The method we describe here addresses both of these aspects: (A) We decided to use ^{3}H- and ^{14}C-radioisotopes for the radiolabeling, as carbon and hydrogen atoms are ubiquitous in organic compounds. Thus, and the incorporation of their radioisotopes allows for the radiolabeling of molecules without alteration of their structures. (B) In order to avoid the difficulties of matching a radiographic image to individual radioactive beads, we use microradiography to visualize the radiation directly at the beads.

In our initial experiments, we acetylated Tentagel-NH_2 beads with ^{3}H- and ^{14}C-labeled acetic acid. The obtained beads had estimated activities of ca. 6500 cpm/bead assuming a nearly quantitative conversion of added acetic acid to resin-bound acetamide. The beads were mixed with non-labeled beads and spread on microscope slides. The slides were coated with a thin layer of molten photographic emulsion and kept overnight at -70 °C in the dark. After treatment of the slides with photographic developing and fixing solutions, radiolabeled beads could easily be distinguished from unlabeled beads under a dissecting microscope as black spots on a clear background.

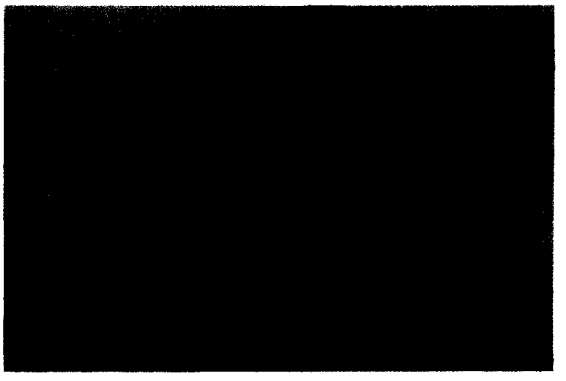
In order to evaluate efficacy and sensitivity of our detection method, we decided to assay **1**, a ^{14}C-radiolabeled analog of a previously described macrobicyclic receptor[11] against a tripeptide library on polystyrene resin beads. The design of the receptor allows for the visual detection of affinity by a red staining of interacting beads and at the same time for microautoradiographic detection through incorporation of ^{14}C-activity, by using 1,4-^{14}C-labeled succinic acid (1.7 μCi/mmol) as linker between the macrobicycle and the dye.



A solution of receptor **1** in chloroform was equilibrated for 24 h with the bead-supported library of acetylated tripeptides. After this time some beads had picked up the red color of the receptor, while the supernatant solution was depleted from the receptor to an extent that it was almost colorless. Aliquots of the bead suspension were plated on microscope slides, embedded in the photographic emulsion as described above, and exposed at -70 °C. Even though the equilibrated beads provided a rather low level of radioactivity due to the low activity of the receptor, we were able to unambiguously localize the radioactive beads after three days. The dark red beads showed a distinct black halo, orange beads have slight halos, whereas the vicinities of colorless beads did not show any signs of exposure to radiation. The long exposure time in this case is due to the weak radiation intensity caused by the low specific radioactivity of receptor **1**.

Assuming that each bead carries 100 pmol of peptide that may interact with our receptor and that on deeply stained beads all of the potential peptide sites on the beads interact with the receptor, we can expect a maximum activity of about 300 cpm/bead. Indeed, we know from earlier experiments that beads having only 10-20% of the peptide sites involved in the interaction with the receptor show intense staining already. Thus, the specific activity of the deeply stained beads in the described assay is probably much lower than 300 cpm/bead, which is therefore a conservative estimate for the sensitivity of the microradiographic identification of radiolabeled beads. Our experiments also show that the intensity of the halos can be easily increased by longtime exposure of the slides at -70 °C. Slides exposed for four weeks show a much stronger staining around radioactive beads without an increased background.

In order to evaluate the potential of our radiographic technique we phosphoimaged slides carrying the equilibrated library. Although the detection with the phosphoimager was faster than by microradiography, the precise identification of individual radioactive spots was made difficult by the high radiation background after overnight exposure that was necessary due to the low specific activity of the beads. Thus, besides the direct visualization of radioactivity at individual beads, the ease of enhancing the microradiographic response without increasing the background is another advantage over phosphoimaging techniques.



Radioassay of the tripeptide library against ^{14}C-labeled receptor 1. The beads binding to the receptor can be clearly identified by the black halo in addition to their red color.

Beads, that have been identified to carry ligands to receptor **1**, were removed individually from the slides. The analysis of the molecular tags encoding the peptide sequences[4] on these dark red radioactive beads revealed specificity for two peptide motifs: (A) a *D*-amino acid followed by *L*-Val and *D*-Gln, and (B) a *L*-amino acid flanked by *L*-Lys and *D*-Pro. These patterns are consistent with those found in the previous screenings of the receptor against the same tripeptide library.[11] Sequences determined from non-radioactive beads did not show any similarity to these motifs.

In summary, we developed a method for the microautoradiographic screening of bead-supported combinatorial libraries with radiolabeled ligands. The radiolabeling of substrates allows for the general tracing of receptor-ligand interactions during "on-bead" affinity assays, and the successful radioassay of receptor **1** shows that the microradiographic conditions do not effect the integrity of our molecular tags nor do they induce artifacts in the screening. The described microradiographic detection scheme promises to be especially advantageous over phosphoimaging based identification of beads in cases where the ligands show only modest discrimination between the members of the screened library or in cases where the individual beads provide only very low radioactivity levels after the equilibration with the radiolabeled substrates.

Experimental Part.

Synthesis of the Encoded Tripeptide Library. The library was synthesized on crosslinked aminomethyl-polystyrene beads (200 mesh, *BACHEM California*) by split synthesis[18,19] using *N*-α-Fmoc-protected glycine, *D*- and *L*-alanine, *D*- and *L*-valine, *D*- and *L*-proline, *D*- and *L*-serine-O-*tert*-butylether, *D*- and *L*-asparagine-*N*-β-tritylamide, *D*- and *L*-glutamine-*N*-γ-tritylamide, and *N*-ε-Boc-*D*- and *L*-lysine. Prior to each amino acid coupling cycle, the encoding photocleavable tags were coupled to the resin using HOBt/DIC to yield 1 pmol of each tag per bead.[4] The amino-termini of the peptides were acetylated by treatment with 5 eq of acetic anhydride and triethylamine, the protecting groups of the functional groups in the side chains were not removed.

Microautoradiographic Screening of 1 against the Tripeptide Library. 3 mg of the encoded tripeptide library were suspended in 400 µl $CHCl_3$. About 10 µl of a 2 mM solution of receptor **1** in $CHCl_3$ were added. The library was equilibrated with the receptor under slight agitation for 24 h. After this period some beads had picked up a dark red color and the solution was almost colorless (supernatant receptor concentration about 40

μM.) Small portions of the suspension of the library were put on cleaned and gelatin coated microscope slides. The spread beads were fixed on the slides with a small volume of a solution of 1 % gelatin in water. After the gelatin solution had dried, the slides were coated in a darkroom with a thin layer of molten *KODAK* NTB-2 emulsion, by dripping the emulsion on the slides and tilting the slides to remove the excess of the emulsion. After the photographic emulsion had resolidified the slides were stored in the dark in a dry atmosphere at -70 °C for 3 days. The slides were treated in a darkroom with *KODAK* Developer D-19 solution (1:1 diluted with water) in water until black dots appeared on the white background. Usually staining could be visually detected after 3-4 min. The slides were washed for 10 s with water and then fixed with *KODAK* Fixer solution in water for 10 min. The processing was finished by washing the slides for 10 min with water. Beads binding to the radioactive receptor **1** could be identified under a microscope (30x magnification) by their red color as well as the black staining of their vicinity. The beads were isolated by softening the gelatin with microliter amounts of water, and picking up single beads with a microliter syringe. The beads were washed five times with anhydrous DMF, and the encoding tags were released and analyzed following the published protocol.[4] The following 21 sequences were determined (amino acids as one-letter codes/*D*-amino acids in lower case, numbers in parentheses show occurrences): pVq (2), qVq (4), kVq (2), aVq, GAq (3), vSq, vqS, kSq, qKq, kNP, KKp, KQp (2), KVp.

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