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NOVEL METHODOLOGY FOR DIFFERENTIATION OF "SURFACE" AND "INTERIOR" AREAS OF POLYOXYETHYLENE-POLYSTYRENE (POE-PS) SUPPORTS: APPLICATION TO LIBRARY SCREENING PROCEDURES

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Because of our interest in devising nonpeptide libraries to be screened by the Selectide process, we explored several ways to generate potential "binding" structures on the receptor-accessible "surface" areas of polyoxyethylene-polystyrene (POE-PS) beads, in concert with appropriate "coding" peptides restricted to the "interior" areas. The overall general concept is to use high molecular weight cleavage, deprotection, or coupling agents (enzymes, polymers) to modify selectively the "surfaces," and to subsequently apply orthogonal peptide synthesis (e.g., Boc and Fmoc chemistries) for the preparation of two different structures on a single bead. The various approaches were evaluated with the model peptides Tyr-Gly-Gly-Phe-Leu (binds to anti-β-endorphin monoclonal antibody), Leu-His-Pro-Gln-Phe (binds to streptavidin), and DPhe-Pro-Arg-Pro-Gly (binds to thrombin). A "blinding" procedure with poly(glutamic acid) covered "surface" areas and prevented model peptides synthesized on "interior" areas from interacting with their receptors. A novel "shaving" procedure involving chymotryptic cleavage provided the desired selectivity in positioning two model peptides between "surface" and "interior" areas.

ENCODED LIBRARIES

Options for the discovery of new drugs have been increased dramatically by the introduction of techniques for the chemical generation and biological screening of so-called combinatorial peptide libraries [1-5]. An essential aspect of these approaches is the efficient elucidation of structures for any active principle recognized and selected by the biological assay system. Initial studies have focused on small peptides built from common amino acids, because such structures can be *both* reliably assembled and readily sequenced [at the scales used in library work, only oligonucleotides share these properties]. However, it is clearly desirable to expand the repertoire of *binding* structures in a library through the incorporation of modified linkages, side-chains, and end-groups which are not found in simple linear peptides and hence cannot be identified by standard sequencing technology. To overcome these limitations, we recently designed and demonstrated experimentally the concept of structural *coding* for non-sequenceable libraries [6]. Thus, our original "one-bead, one-peptide" principle [Selectide process described in ref. 2] is extended to "one-bead, two-structures." The synthetic strategy and randomization procedure is such that each sequenceable coding structure (a peptide) is correlated with a non-sequenceable structure on the same bead that will be screened for binding; biologically active structures are then deduced from the conjugate coding sequence. The "code" can have multiple "letters" corresponding to a single component of the binding structure. Independent of our work, ideas for encoding by peptides or relatedly by DNA (with later PCR amplification) have been proposed by Kerr *et al.* [7] and Brenner and Lerner [8], respectively. In all of these approaches, the coding and screening sequences are created at essentially equimolar levels and are both present contiguously throughout the bead. This raises the possibility for ambiguous interpretations should the coding sequence bind to the macromolecular receptor target, or should some "cooperative" interaction between the two structures on the bead lead to a positive biological result. Such outcomes might be addressed if the screening structures are

synthesized onto a selectively cleavable linkers; a potentially active binding structure can then be released and the bead can be retested [9].

BEAD DIFFERENTIATION

In the present work, we introduce the idea of *physically* separating the coding and screening sequences that are present on the same bead. The goal is to identify and exploit different physical characteristics of "surface" and "interior" areas, in such a way that only the screening structure is present on the surface (hence available for possible biological interactions), whereas the coding sequence is restricted *exclusively* to the interior. As can be shown from literature considerations and our own preliminary experimental results, substantially less than 1% of the total functional sites in typical microporous beads are receptor-accessible, so this plan dictates that the overwhelming majority of structures assembled on each bead are the interior coding sequences. It is not particularly detrimental for some of the screening structures to be also present within the interior areas, so long as sequencing carried out on beads that show a positive biological result gives an unambiguous readout of the coding structure. The desired differentiation between surface and interior will be established usually *prior* to syntheses of the two classes of structures, although one can also envisage variations where *post-synthetic* reactions effect the selective removal or destruction of appropriately linked or designed coding structures from the surface.

As detailed below, two general kinds of approaches were considered for selective surface modification. One tactic aimed to set up barriers to chemical reactions, by taking advantage of immiscible solvent systems, solvent phase changes, or physical barriers. Alternatively, we anticipated that reactions, e.g., acylation, deprotection, or proteolysis, that are mediated by *macromolecular reagents*, i.e., functionalized polymers, modified proteins, and enzymes, could be restricted to the surface areas. A useful clue for the promise of this latter approach is provided by the very low capacities of microporous beads for conjugation of small proteins, consistent with surface immobilization [10]. In contrast, relatively small molecules penetrate uniformly throughout the interior of beads [11]. It should be stressed that bead "surface" area is dynamic, defined by the molecular weight, shape, and flexibility of the probing reagent, as well as properties of the bead.

Integrated into our approaches is the identification of suitable beaded polymers. Requirements include: (a) relatively uniform macroscopic size and morphology; (b) proper microporous architecture; (c) adequate mechanical stability; (d) compatibility with organic reagents and solvents for efficient peptide synthesis throughout the bead; and (e) hydrophilic character compatible with selective surface modifications and biological testing carried out in aqueous milieus. In our experience, commercially available polyoxyethylene-polystyrene (POE-PS) graft supports have the proper combination of physicochemical properties [10]. At the moderate initial substitution level used, i.e., 0.2 to 0.3 mmol/g, the size, solvation, and swelling properties of these supports are relatively unaffected by pendant growing peptide chains. The POE portion of POE-PS may also serve as a spacer to improve the accessibility of binding structures to their interacting targets.

GENERAL EXPERIMENTAL DESIGN

The feasibility studies reported herein are pre-requisite to our ultimate goal to apply bead differentiation technology for the production and evaluation of encoded libraries. The current objective was to prepare beads bearing any two biologically active model peptides positioned so that *only* the "surface" peptide would interact with its receptor. Moreover, the hope was for the "interior" peptide to *not* interact with its receptor, despite being the predominant species on the bead as evidenced by analytical and sequencing data. Whenever both peptides on the bead were detectable by their respective receptors, it was concluded that a given bead differentiation approach had failed. The three model peptides chosen [Leu-His-Pro-Gln-Phe which binds to streptavidin, Tyr-Gly-Gly-Phe-Leu which binds to anti-ß-endorphin monoclonal antibody, and DPhe-Pro-Arg-Pro-Gly, which binds to thrombin] are each small, relatively straightforward to synthesize, and involved in specific, high-affinity binding interactations with macromolecular receptors.

The described work was carried out with TentaGel AM beads (130 μm, 0.21 mmol/g, Rapp Polymere, Tübingen, Germany), which were extended with a ßAla-Gly-ßAla-Gly spacer similarly to our earlier library studies [2, 12]. Next, a substrate for the key surface modification step (examples and details follow) was introduced, by either standard N^{α}-*tert*-butyloxycarbonyl (Boc) or N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) chemistry, with DIC/HOBt-mediated coupling and

bromophenol blue monitoring [13]. Selective deprotection or cleavage was designed to expose free amino groups (ideally just on the surface), which were the starting point for assembly of the first peptide by several cycles of chemistry orthogonal to that used to provide the original substrate. After assembly of the first peptide was complete (retaining orthogonal protection on the *N*-terminus), the original substrate (ideally now restricted to the interior) was deblocked, and the second peptide was built up with chemistry corresponding to the original protecting group. Orthogonal Fmoc followed by Boc synthesis was carried out with Mtr, Trt , and *t*Bu respectively for Fmoc-Arg, His, and Tyr side-chain protection, followed by Tos, Dnp, and H for Boc-Arg, His, and Tyr. Alternatively, orthogonal Boc followed by Fmoc was with Tos, Boc, and H for Boc-Arg, His, and Tyr, followed by Pmc, Trt, and *t*Bu for Fmoc-Arg, His, and Tyr side-chain protection. Appropriate final deprotection procedures now gave beads suitable for biological evaluation. More specifically, in the Fmoc followed by Boc case, sequential deprotection was carried out with thiophenol–DMF (1:9), 1 h at 25 °C; reagent K, TFA–phenol–thioanisole–water–1,2-ethanedithiol (82.5:5:5:5:2.5), 1 h at 25 °C (if no Mtr) or 2 h at 50 °C (if Mtr present); piperidine–DMF (1:1), 20 min at 25 °C; and dilute aqueous HCl (to protonate free amino group); in the Boc followed by Fmoc case, reagent K and piperidine–DMF, either order, were followed by HF–anisole (9:1), 1 h at 0 °C.

The synthetic scheme outlined in the preceding paragraph is most suited for model experiments, since the two test peptides are assembled consecutively. The indicated stepwise approaches were preferrable to segment condensation alternatives in which the preformed *N*-protected peptides were attached directly. While involving less labor, segment condensation in these cases suffered from slow rates, and overall yields in the less than desirable 40 to 60% range. For the eventual application to encoded libraries, stepwise "zig-zag" syntheses will be necessary, with alternating cycles of Fmoc and Boc chemistries to incorporate residues respectively for the surface screening and the interior coding structures. Working with the model peptides, the consecutive and the zig-zag approaches were both shown to provide effective syntheses of the desired sequences.

ATTEMPTED BARRIER APPROACHES

Several ideas were examined with the goal of area-selective removal of the Boc group. For example, beads were soaked in 20% aqueous NaCl at -15 °C, rapidly aspirated to form a wet bed, and pulsed (3 to 60 sec, once or twice) with TFA–CH_2Cl_2 (1:4 to 1:1) at -15 to +25 °C. Reciprocally, beads were swollen in DMF and deblocked with concentrated aqueous HCl (10 to 60 sec) at -60 to 0 °C. These studies were based on the premise that the first-mentioned solvent milieu would shield internal areas from the immiscible deprotecting reagent. Alternatively, water was *frozen* inside the beads in an attempt to create a diffusion barrier against cold TFA–CH_2Cl_2 (1:4 to 1:1) applied (10 to 45 sec) for Boc removal. Depending on the parameters of reaction design, temperature, time, and deprotective reagent concentration, this array of experiments revealed levels of cleavage ranging from 5 to 40%. Unfortunately, the one-bead, two-test peptide model system gave no evidence for significant differentiation of surface versus interior areas. Furthermore, these approaches suffered from severe mechanical complications that impact on uniformity and reproducibility, i.e., beads retained the phase behavior of the interior solvent, and clumped together so that separation of individual beads was difficult or impossible. Still under investigation are similar studies involving base-promoted Fmoc deprotection, or relatively short photolysis of 2-(3,5-dimethoxyphenyl)-propyl[2]oxycarbonyl (Ddz) and other photolabile protecting groups.

SELECTIVE SURFACE MODIFICATION BY "BLINDING"

We define "blinding" as the selective coupling of a macromolecular reagent to the surface of a bead, in a way that does not interfere with further chemical synthesis in the interior regions. Ideally, the blinding process should be reversible; chemistries to achieve this are readily envisaged but have not yet been shown in practice. In the prototype experiment to demonstrate blinding, the resins were solvated in a pH 5.7 aqueous buffer and reacted with varying amounts of poly(glutamic acid) (MW 30 kDa; Sigma Chemical, St. Louis) in the presence of the water-soluble coupling agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide·HCl (EDC). Next, unreacted carboxyl groups were capped by EDC-mediated coupling of ethanolamine. Later, the model peptide Tyr-Gly-Gly-Phe-Leu-Gly-Gly-Gly [the triglycyl tail was added to improve accessibility to the receptor] was introduced by the usual Fmoc protocol, and beads were tested by the standard assay with anti-β-endorphin. Coupling of sufficient poly(Glu) during the first phase of the experiment did indeed block the antibody interaction (Table 1, bottom line). This was despite the fact that the level

Table 1. Staining Reaction of Tyr-Gly-Gly-Phe-Leu-Gly-Gly-Gly Synthesized on Beads After "Blinding" by Poly(Glutamic Acid)

Poly(Glu) mg/0.2 mL resin	Relative anti-ß-endorphin reactivity	Peptide/Bead (quantitative Edman)
0	3+	72 pmol
0.08	2+	N.D.
0.4	1+	N.D.
10	trace	65 pmol

N.D. means "not determined."

of peptide assembled onto the beads (10 selected at random and combined for sequencing) was essentially the same for the control and the fully blinded cases (per bead: 72 pmol vs. 65 pmol). These studies are consistent with derivatization of all available surface amino groups upon poly(Glu) treatment, or alternatively with physical interference by a poly(Glu) layer to prevent any interaction between the antibody and the interior model peptide.

SELECTIVE SURFACE MODIFICATION BY "SHAVING"

The term "shaving" refers to a chemical or enzymatic treatment that leads to the selective exposure of functional groups on the surface of a bead. Initial attempts to achieve this used Jeffamine [MW 6 kDa, a bifunctional POE with amino endgroups] or poly(ethyleneimine) [PEI; MW 50 to 60 kDa] as macromolecular reagents for Fmoc removal. Deblocking with Jeffamine (20 to 50% w/v) in DMF was relatively slow, both for solution reaction on Fmoc-Gly and for the reaction of Fmoc-Gly-TentaGel. This approach was abandoned due to insufficient discrimination observed between solution and solid-phase rates, as well as the possibility that the actual deprotecting agent may be dimethylamine formed by base-catalyzed decomposition of DMF. Turning to PEI (10% w/v) in a variety of solvents, we found that solution deprotection in DMF or DMSO was complete within 2 min at 25 °C, whereas deprotection in absolute ethanol required 1 h for completion. Transferring these conditions to Fmoc-Gly-TentaGel, the rate of Fmoc removal by the viscous PEI solutions was definitely retarded, but could be accelerated by vigorous stirring and/or ultrasonic agitation. The one-bead, two-peptide biological test system gave no evidence for surface/interior differentiation from PEI-mediated deprotection. Results were the same when the polymer was purified by gel filtration to remove low molecular weight amines. We suspect that traces of water adsorbed to the polymer result in the production of hydroxide, which can diffuse throughout the bead to effect deprotection. We still consider the overall concept valid, and are planning to examine other combinations of protecting groups and polymeric deprotecting agents.

Subsequent experiments focused on the idea of using enzymes as macromolecular "shaving" agents. Typical proteolytic enzymes are in a size range (MW 20 to 50 kDa) that is expected to limit diffusion inside microporous beads. Furthermore, a number of enzymes (a) are readily available; (b) show good stability; (c) function under ambient aqueous conditions; (d) provide efficient catalysis; and (e) exhibit good substrate specificities. We incubated several peptide-resins with chymotrypsin [per 0.2 mL of settled polymer beads, 1 mg chymotrypsin in 1 mL of 0.1 M ammonium carbonate, pH 7.8, 37 °C, 20 h; repeated twice for 4 h each with fresh enzyme in buffer] and tested the reactivities of the resultant beads with appropriate macromolecular acceptors (Table 2). Gratifyingly, it was possible to abolish all binding activity of beads bearing Tyr-Gly-Gly-Phe-Leu to anti-ß-endorphin antibody, even though quantitative Edman degradation revealed that the loading of peptide on the beads was unaltered by the proteolysis step. On the other hand, chymotrypsin failed to remove completely Leu-His-Pro-Gln-Phe from the surface, consistent with this particular streptavidin-binding sequence being a poor substrate for the enzyme. A simple Tyr-Gly or Trp-Gly linker at the *C*-terminal of the model pentapeptide created a better chymotrypsin substrate, and now the "shaving" procedure eliminated the interaction with streptavidin.

The aforementioned results encouraged us to use "shaving" with chymotrypsin as the key step to establish surface/interior differentiation according to the one-bead, two-peptide concept explained earlier. Resins with the ßAla-Gly-ßAla-Gly spacer were extended with Boc-Phe, Tyr, or Trp, and then incubated with the enzyme. Relatively small amounts of the Boc-amino acids were released (difficult to quantitate). The resultant "shaved" resins were then either "capped" by acetylation

Table 2. Staining Reaction of Various Peptide-Beads, Before and After "Shaving" with Chymotrypsin

Peptide on bead	Binding to			
	Anti-ß-endorphin		Streptavidin	
	Control	"Shaved"	Control	"Shaved"
Tyr-Gly-Gly-Phe-Leu	5+	0	0	N.D.
Leu-His-Pro-Gln-Phe	N.D.	N.D.	3+	2+
Leu-His-Pro-Gln-Phe-Gly	N.D.	N.D.	3+	0 to 1+
Leu-His-Pro-Gln-Phe-Tyr-Gly	N.D.	N.D.	3+	0
Leu-His-Pro-Gln-Phe-Trp-Gly	N.D.	N.D.	3+	0

(0.3 M *N*-acetylimidazole in DMF for 20 min), or acylated by Fmoc-Leu, Phe, or Gly [the respective *C*-terminal residues of the three model peptides]. Subsequently, the necessary cycles of Fmoc chemistry were carried out to assemble the model peptides, which were hopefully confined to the surface areas of the beads. Through these syntheses, the levels of Fmoc released at each deprotection were quantitated by UV spectroscopy, and corresponded from 1 to 2% of the total amino sites originally on the resins. As an estimate for the extent of "shaving," the values given are only approximate, since they approach the sensitivity limits of the UV technique. After completion of the Fmoc syntheses, the Boc group protecting the majority of amino acyl-spacer chains on the resins was cleaved, and Boc chemistry was used to build a different model sequence, presumably in the "interior" areas. Final deprotection gave the beads containing two peptides, suitable for analytical work and biological testing (Table 3). The data show clearly that as a consequence of the "shaving" procedure: (a) the "interior" peptide structure was read out by sequential Edman degradation, and residues corresponding to the "surface" peptide were found only in trace or undetectable amounts; (b) the synthesized "surface" peptides were detected readily by their receptors, for all three model peptides; (c) the synthesized "interior" peptides were not detected by the receptors in the cases of Leu-His-Pro-Gln-Phe and DPhe-Pro-Arg-Pro-Gly, although the interaction between Tyr-Gly-Gly-Phe-Leu and the anti-ß-endorphin antibody was so strong that it was observed despite "shaving"; (d) results were most clear-cut when initial shaving was carried out with the superior chymotryptic substrate Boc-Trp-Gly; experiments with Boc-Tyr-Gly were qualitatively similar but complicated by the possibility of peptide growth of the unprotected phenolic side-chain, and studies with Boc-Phe-Gly were abandoned due to the lesser level of enzymatic

Table 3. Staining Reaction of Various Beads Bearing Two Peptides Assembled After "Shaving" of Boc-Trp-Resin with Chymotrypsin[a, b]

No.	Peptide in Bead Area		Binding to		
	"Surface"	"Interior"	Anti-ß-endorphin MoAb	Streptavidin	Thrombin
1	*N*-acetyl	LHPQFWG	0	0	N.D.
2	*N*-acetyl	YGGFLWG	3+	0	N.D.
3	*N*-acetyl	fPRPGWG	N.D.	N.D.	0
4	LHPQFG	YGGFLWG	3+	5+	N.D.
5	YGGFLG	LHPQFWG	4+	0	N.D.
6	fPRPGG	LHPQFWG	N.D.	0	3+
7	LHPQFG	fPRPGWG	N.D.	3+	0

[a] In setting up this Table, the one-letter code for amino acids has been used in order to conserve space. "Surface" and "interior" bead areas are defined operationally based on the experimental design involving chymotryptic "shaving," as described in the text. N.D. means "not determined."

[b] Quantitative Edman degradation was carried out on the beads from lines 4 and 5. The readout clearly gave the sequences corresponding to the peptides designated as "interior" (93 to 114 pmol at first cycle, 68 to 73 pmol at fifth cycle). At the same time, PTH derivatives corresponding to readout of the designated "surface" peptides were absent or present in trace amounts (<1%); the only exception came with Phe (4 to 5 pmol) attributed to preview or lag of the major "interior" peptide.

cleavage. The Tyr-Gly-Gly-Phe-Leu – anti-β-endorphin antibody system was studied separately (see below); however, our original surface/interior screening/coding concept is supported in the Leu-His-Pro-Gln-Phe – streptavidin / DPhe-Pro-Arg-Pro-Gly – thrombin systems (either order; refer to Table 3, lines 6 and 7).

Finally, we report the results of experiments designed to probe the reason for the "false positive" observed when the Tyr-Gly-Gly-Phe-Leu peptide binding the anti-β-endorphin antibody was supposedly confined to the interior areas of the beads (see Table 3, lines 2 and 4). The amounts of pentapeptide synthesized on surface areas of the beads were limited by a protocol in which, after shaving, defined mixtures of Fmoc-Gly and Boc-Gly were coupled (i.e., 1:0, 1:1, 1:9, and 1:99). There followed Boc deprotection, acetylation, and synthesis of the model pentapeptide by Fmoc chemistry. Even when Tyr-Gly-Gly-Phe-Leu was present on only 1% of the surface sites (corresponding to an estimated < 0.02% of the total sites in the bead), it could be detected by the antibody interaction. The levels of peptide on any of these beads were too low to be detected by amino acid analysis or Edman degradation sequencing of single beads. Extrapolating this sensitivity level to the earlier studies, >95% enzymatic shaving of the surface would still leave ample surface sites bearing the putative "interior" peptide.

CONCLUSIONS

We have defined an effective enzymatic "shaving" system that can allow differentiation between surface and interior areas of POE-PS beaded supports. In studies carried out to date, such a differentiation could not be achieved by the use of low molecular weight reagents in conjunction with physical or phase barriers, nor with macromolecular chemical reagents. The enzymatic approach requires a good substrate for shaving, appropriately matched molecular sizes for the enzyme and the biological receptor, and appropriate affinities of the receptor to its ligand. The successful variations hold substantial promise for applications to encoded libraries.

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