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Photoactive spatial proximity probes for binding pairs with epigenetic marks

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ABSTRACT

A new strategy for encoding polypeptide libraries with photolabile tags is developed. The photoassisted assay, based on conditional release of encoding tags only from bound pairs, can differentiate between peptides which have minor differences in a form of post-translational modifications with epigenetic marks. The encoding strategy is fully compatible with automated peptide synthesis. The encoding pendants are compact and do not perturb potential binding interactions.

1. Introduction

High throughput combinatorial chemistry is critical for modern drug discovery, with parallel synthesis and screening techniques currently being the industry standard. More than two decades after the introduction of massively high-throughput one-bead-one-compound split-and-pool combinatorial methods [1,2], *parallel synthesis and screening still dominate the landscape of big pharma.* Hybrid methodologies, such as synthesis in NanoKan containers have been adopted by some companies to take advantage of polymeric bead-supported synthesis while keeping track of the identity of individual compounds being synthesized in the barcoded NanoKans.

When used, one-bead-one-compound libraries are normally assayed for binding of biological targets through fluorescence-guided mechanical segregation of the winning beads. The required mechanical manipulations impose a lower limit on the size of the particle used as a solid support. Currently there is no simple way for assaying solution phase mixtures. While various iterative deconvolution methods [3] have been developed to synthesize and screen soluble sub-libraries, all of them require multiple redundant synthetic steps, are time consuming, and expensive. Solution phase combinatorial chemistry holds great promise, as it is compatible with both divergent and convergent multi-step synthetic schemes, and not constrained to linear synthesis [4]. Its immense potential, however, has not yet been fully realized, partly due to the complexity of assaying solution mixtures for binding.

We note that there are currently problems for which (i) on-bead screening is not possible at all, and (ii) parallel approaches are, if not impossible, then prohibitively time-consuming and labor-intensive. One of these problems is high throughput screening of combinatorial libraries for binding between individual members of the library. As an example, understanding homo- or hetero-dimerization, -trimerization and formation of higher order oligomeric assemblies of peptides is of utmost importance in the field of structural biochemistry for a variety of reasons, ranging from fundamental insights into protein folding, protein–protein interactions including aggregation, to the applied considerations of building artificial self-assembled molecular architectures and de novo protein design. Huge combinatorial libraries of peptides are readily available via parallel and especially split-and-pool synthesis on polymeric beads. However, there are no high throughput methods to rapidly screen such libraries for oligomers possessing the tightest di-, tri-, etc. hetero-oligomerization binding constants.

Potentially, one can use parallel libraries to test for such interactions, but the number of possible combinations quickly becomes prohibitively high – for a library of N compounds one would have to conduct N^2 binding experiments for the dimers, N^3 – for the trimers and so on. For example, in a very modest library of only 625 peptides the number of all possible dimers exceeds 390,000, the number of

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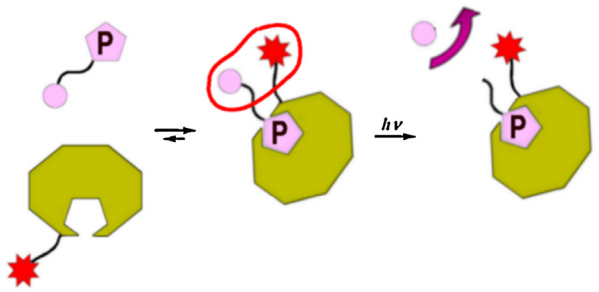

Scheme 1. Externally sensitized photorelease of tags is contingent on molecular recognition.

trimers exceeds 244 million and the number of tetramers exceeds 150 billion.

Various approaches are used to test for interactions between biomolecular entities in solutions: differential scanning calorimetry, titration calorimetry, analytical centrifugation, spectroscopy including NMR, plasmon resonance, and mass spectrometry, of which only the latter has enough "bandwidth" to deal with modest combinatorial collections of biomolecules. Detection of non-covalent interactions between biological molecules by ESI mass-spectrometry was pioneered in early 90s, most prominently by Ganem [5a,5b](for a review see [5c]). Later MALDI-TOF methods followed [6]. It is still not clear whether or not gas phase affinities, measured by these methods, reflect the actual binding in solution.

Yet, while current analysis is not ideal, the comprehensive understanding of the fundamentals of peptide–peptide interactions is at the core of a number of critical problems in biomedical sciences. One specific problem concerns diseases involving aggregation of peptides or small proteins, especially such neurodegenerative disorders as Alzheimer's disease, Huntington's disease etc. [7]. It is not surprising then that the studies directed toward better understanding of protein folding (for example in the DeGrado [8] or Gellman [9] labs) are always intertwined with studies of peptide–peptide interaction and oligomerization. Unfortunately, the progress in the *rational design* of short peptides capable of forming dimers or trimers is at best spotty. There are few prominent examples of this in the literature, including one from the Imperiali lab [10]. Combinatorial approaches were designed to augment rational design, especially for cases of great complexity where theories are lacking or deficient. If there were a high throughput method to test for peptide–peptide interactions one would obtain invaluable information about the very basic motifs of such intermolecular binding. Regrettably, there are no methods currently available to test for such interactions in a truly high-throughput manner.

We have recently developed a method for chemical encoding of combinatorial libraries with photolabile externally sensitized tags, which will allow us to pre-screen solution phase libraries for bound dimers or higher oligomers or, in more complex cases, to dramatically narrow the possibilities to a manageable subset of potential candidates. Our tagging methodology makes use of dithiane-based photolabile molecular systems that are capable of photoinduced fragmentation *only when in the presence of an external sensitizer*. The mechanism of such fragmentation has been shown to involve oxidative single electron transfer (ET) from the dithiane moiety to the excited (most commonly, triplet) ET-sensitizer followed by a mesolytic fragmentation in the generated cation radical [11]. In these systems, photoinduced fragmentation is contingent upon the occurrence of a molecular recognition event. In other words, a molecular recognition event is needed to *arm* the system, after which it becomes photolabile.

Scheme 1 outlines the general concept of such a system: one component of the molecular recognition pair (green octagon), is outfitted with a compact ET-sensitizer, e.g. xanthone, whereas the dithiane adduct is tethered to the second component (pink pentagon labeled P). Binding brings the sensitizer into the vicinity of the tag adduct, at which point irradiation commences. Xanthone sensitizes fragmentation in the adduct, releasing the dithiane tag into solution. The tags are then detected in a standardized analytical protocol revealing the identity of the bound pairs. A diverse set of substituted dithiane tags is readily available for encoding. Additionally, to further diversify the available variety of tags, we have identified non-dithiane encoding tags, for example derivatives of trithiabicyclo[2.2.2]octanes, which can be used in combinatorial encoding [12].

Tagged combinatorial libraries are well precedented. In the classical bead-tagging approach, for example the one developed by Clark Still [13], each bead is encoded with the full set of tags needed for subsequent identification of the ligand displayed on the surface of the bead. Still's strategy is not suitable for encoding individual molecules, which simply do not have enough "real estate" to accommodate all the encoding tags without running into a risk of multiple tags interfering with molecular recognition events. Our strategy is to encode individual molecules one tag at a time. Every library member can be encoded with a set of tags so that a certain fraction of its molecules are encoded with the first tag, another fraction – with second, etc., with the net result of each ligand being present in the solution as several sub-populations cumulatively encoded with all the tags necessary for its subsequent identification. Irradiation in this case yields the desired result because all the tags encoding individual molecules in the bound pairs are collectively released into the solution, where they are analyzed revealing the identity of the bound compound.

Initially we proved this general concept of tagging the individual (unsupported) library members and screening for molecular recognition using the known binding pair, avidin–biotin [14]. In these experiments a mini library of compounds, which included biotin, was encoded by tagging each compound with different dithianes. Scheme 2 gives a general outline for screening of such library of ligands (biotin is depicted by a pink "B" pentagon), encoded with the tethered tags (pink circles 1–5). Avidin (the green octagon "A") is outfitted with a xanthone-based ET-sensitizer, and incubated with the library. The binding event brings the sensitizer into the vicinity of the tag/adducts (encircled with red), which, upon irradiation triggers the release of tags 1–5, i.e. only the tags which encode biotin.

So far our dithiane tags-encoding methodology was tested with a model barbiturate-binding artificial receptor [15] and validated for the avidin–biotin binding pair [14], known to have a very tight K_D. In this study we extend this approach onto biological systems exhibiting low micro molar affinities and demonstrate that it can be used to differentiate between very similar epitopes modified via post-translational modifications (PTMs), such as unmodified and methylated lysine. Our choice of the protein substrate is ING2 PHD (plant homeodomain) finger, which is known to recognize a hexapeptide fragment of the histone H3 tail Ala-Arg-Thr-Lys-Gln-Thr, but only when the lysine residue is trimethylated, i.e. Ala-Arg-Thr-Lys(Me3)-Gln-Thr. The trimethylated epitope is referred to as H3K4me3.

Nuanced aspects of molecular recognition play a pivotal role in biological processes. This is especially important for the emerging field of epigenetics, where PTMs regulate complex signaling cascades, including gene transcription, DNA repair, recombination, and replication and chromatin remodeling [16,17]. Epigenetic misregulations have been linked to various human diseases including cancer, premature aging and neurodegenerative disorders, and thus development of experimental approaches to characterize PTM recognition is essential in understanding the epigenetic mechanisms, PTM-driven functional outcomes, and disease-associated