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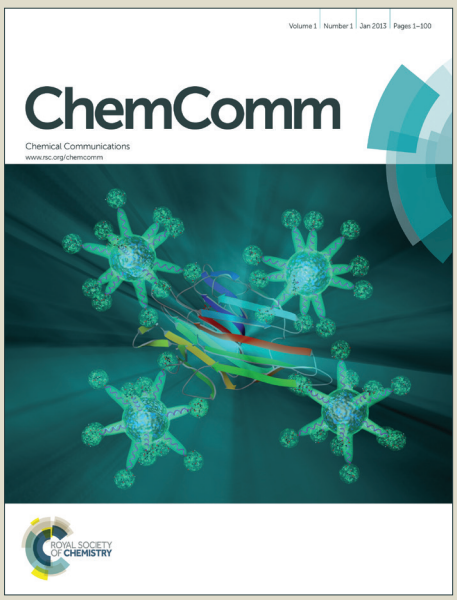

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A Time-Resolved Luminescence Biosensor Assay for Anaplastic Lymphoma Kinase (ALK) Activity†

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Wei Cui[a] and Laurie L. Parker‡[a]

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A novel time-resolved luminescence biosensor assay for anaplastic lymphoma kinase (ALK) was developed. We used a straightforward strategy to modify a known ALK substrate into a peptide biosensor that can accommodate terbium luminescence sensitization upon its phosphorylation by ALK. Since this strategy is generalizable, this high-throughput screening compatible assay serves as an example for development of other kinase assays that employ terbium luminescence as a read-out.

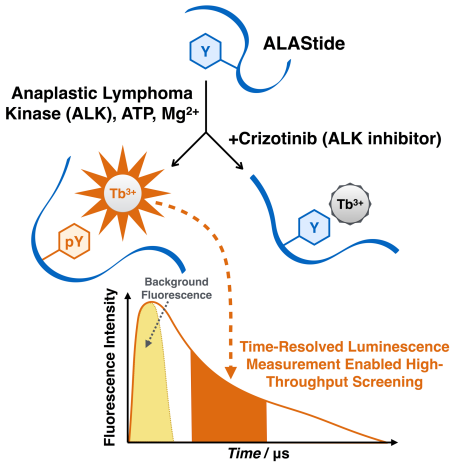
Multiple types of cancer are driven by the abnormal activity of protein tyrosine kinases. The anaplastic lymphoma kinase (ALK) is one such enzyme that, when modified via genetic translocation and other mutations, becomes an aggressive oncogene[1]. ALK fusion kinases[1], and one fusion in particular, the EML4-ALK fusion, can lead to the onset of different types of cancer[2–4]. Resistance is a major clinical concern for the ALK inhibitor, crizotinib[2,6]. New generations of ALK inhibitors (such as LDK378[7,8]) are being actively developed, yet limited progress has been achieved so far.

One key factor to facilitate the drug development process for ALK is efficient and low-cost high-throughput screening (HTS) assays designed for the drug target. Currently, enzymatic assays for kinases like ALK are available that use radioactive, immunological[9], or absorptiometric detection[10,11] (the latter of which uses a tandem reaction for indirect monitoring that requires extra enzymes including pyruvate kinase, lactate dehydrogenase and NADH). While these methods serve as helpful tools, they still suffer from several limitations that affect their implementation in drug screening, including a) low signal to noise ratio and background interference that could compromise the sensitivity and specificity of the assay, and b) the usage of radioactive material, antibodies, or enzymatic tandem reactions that lead to both higher cost and potentially lower throughput and reproducibility issues. Time-resolved luminescence detection can exclude the background signal from the complex biological environment and enable significant improvement in signal to noise ratio when compared with steady state fluorescence[21], and has been combined with Förster resonance energy transfer (FRET) in order to provide improvements (e.g. the LanthaScreen™ assay from Life Technologies). However, such antibody-based FRET techniques still require fluorophore-labeled substrates and Tb^{3+}-chelator conjugated antibodies, in which Tb^{3+} has no direct interaction with the phosphorylation site and provides only an indirect measurement.

As a strategy to overcome such problems, peptide-based approaches can be integrated into various analytical workflows for developing HTS compatible kinase assays[12–15]. Several examples of detecting tyrosine phosphorylation directly by lanthanide luminescence (rather than the antibody-dependent technologies) have been demonstrated[17–20], either by using sensitizer-labeled peptides or peptides with inherent lanthanide-sensitizing sequences. Because they don't require any special labeling, the latter types are chemically easier to prepare—however, they are also more challenging to design since the balance between substrate specificity, efficiency, and lanthanide chelation must be carefully considered. Most known kinase substrate peptides are not effective at chelating and sensitizing lanthanides, so there is a need for generalized approaches that can be applied to adapt well-characterized kinase substrates for lanthanide luminescence read-outs.

We previously employed time-resolved lanthanide luminescence to design a high-throughput screening (HTS) compatible biosensor assay (Scheme 1) for Syk kinase activity[15]. However, lanthanide sensitization by the Syk substrate we developed was fortuitous based on the inherent substrate sequence, and not necessarily applicable to other substrates, which may not have the same advantageous chelating residues. Here, we adapted that strategy to design a

novel peptide biosensor ALAStide (ALK Artificial Substrate peptide) and developed a HTS-compatible ALK assay using time-resolved luminescence as the detection method (Scheme 1). ALAStide is based on a previously characterized ALK substrate[9], but directly sensitizes terbium luminescence once phosphorylated, and thus avoids many of the pitfalls and limitations of other antibody-based homogenous time-resolved fluorescence (HTRF) assays. The strategy of our design can be easily expanded to other drug-target kinases as well.



Scheme 1. ALAStide Detects ALK Activity and Inhibition by Sensitization of Time-resolved Luminescence.

The Y1278 autophosphorylation site within the ALK kinase domain A-loop has previously been used to develop a peptide substrate (termed YFF) with a radiometric ALK assay[9], but radioactive kinase assays are typically not HTS compatible. The YFF peptide sequence cannot be used directly for lanthanide sensitization due to its lack of appropriate amino acids for metal chelation (Figure 1a). Therefore, we modified specific residues of ALK Y1278 fragment to confer the required chelating properties for a Tb^{3+} sensitizing substrate[16,17] by using the available information about ALK substrate positional selectivity[9]. Existing tyrosine-centered Tb^{3+} binding sequences usually have hard base-containing ligand amino acids (e.g. E or D) flanking the center tyrosine[17]. To determine an appropriate sequence for our biosensor, we introduced point mutations to an eicosapeptide corresponding to an expansion of the YFF mutation of the fragment of ALK surrounding the Y1278 site (Figure 1a). Point mutation positions were chosen based on previously reported alanine scan studies[9]. For the ALK Y1278 fragment, the region of +4 to +7 has been shown to be important for autophosphorylation[9]; therefore we restricted the mutation sites to the -7 to +3 region. We first introduced two aspartic acid residues in the N-terminus of Y1278 sequence to serve as potential Tb^{3+} ligands. The -3 to -1 region was not changed, since it represents a conserved region in the A-loop of all kinases in the IRK family[9,22]. For the +1 to +3 region, it has been shown that charged mutations are not tolerated by ALK, while hydrophobic mutations do not affect the catalytic efficiency[9].

To investigate the acceptability of chelation-compatible mutations in the +1 to +3 region, +2D and +2M mutations were synthesized and tested in the context of the -6D, -4D mutations (Figure 1a) with recombinant active ALK kinase domain and ELISA-based detection. We found that while +2D (Substrate A in Figure 1a) completely prevented substrate phosphorylation, +2M fortuitously showed a significant increase in phosphorylation rate compared to the original sequence (Figure 1b). We also found that polar mutations (+2N and +3T, Substrate B in Figure 1a) resulted in lower phosphorylation than the reference peptide. Additional -1H mutation (Substrate C in Figure 1a) completely prevented phosphorylation, indicating the importance of the conserved -3 to -1 region. The -6D, -4D, +2M substrate (ALAStide) showed a similar specificity profile against other tyrosine kinases as the natural sequence and the YFFtide[9] (Figure 1c). We found that neither the reference peptide nor ALAStide were appreciably phosphorylated by Lyn, Abl or Csk (Figure 1c), while both were phosphorylated by Src *in vitro* (Figure 1c, top right panel). This may suggest that Src participates in ALK activation and/or downstream signaling, however further investigation will be needed to determine the biological relevance of this phenomenon. Nevertheless, since methionine can also serve as a Tb^{3+} ligand[17], this set of mutations managed to introduce ligand amino acids on each side of the tyrosine while providing an efficient, specific, and partially selective ALK substrate suitable for *in vitro* kinase assays.

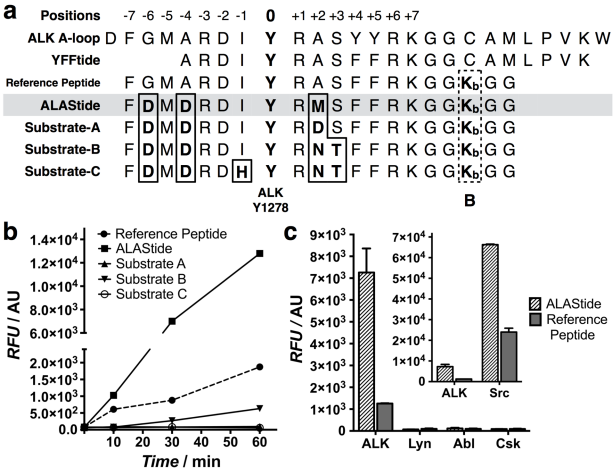


Figure 1. Design of a novel ALK substrate, ALAStide. (a) Sequence comparison of the ALK activation loop (A-loop), the YFF peptide[9], the reference peptide used in this research (which is a hybrid of the YFF peptide and further extensions into the A-loop) and novel mutations resulting in ALAStide. Position 0 refers to the Y1278 site. ALAStide is shown with grey background. "**B**" and dashed box indicate biotinylated lysine (K_b) that is required for ELISA experiments. Solid lined-boxes indicate mutations tested during the development of ALAStide. (b) Phosphorylation of ALAStide and the reference peptides by ALK were evaluated by ELISA detection. (c) Phosphorylation of ALAStide and the reference peptide by representative tyrosine kinases was evaluated by ELISA detection after 30 min reactions. Data in (c) indicate

averages ± SEM for triplicate experiments. RFU: Relative Fluorescence Unit. AU: Arbitrary Unit.

To examine the success of our design strategy, both phosphorylated (pALAStide) and unphosphorylated (ALAStide) biosensors were prepared and tested for their capacity to sensitize time-resolved Tb^{3+} luminescence. The excitation wavelength was set to 266 nm for both peptides. After a 50 μs delay, time-resolved luminescence spectra between 450 nm and 650 nm were recorded for 1 ms. Strong Tb^{3+} sensitization was observed for pALAStide in the presence of Tb^{3+} (Figure 2a), whereas unphosphorylated ALAStide showed no difference in luminescence from buffer containing only Tb^{3+}, indicating that on its own, ALAStide does not substantially chelate or sensitize the Tb^{3+} ion (Figure 2c). As a result, a 5.5 fold increase in luminescence intensity of the phosphorylated form compared to the unphosphorylated form was achieved, providing the analytical foundation for a time-resolved luminescence assay (Figure 2a).

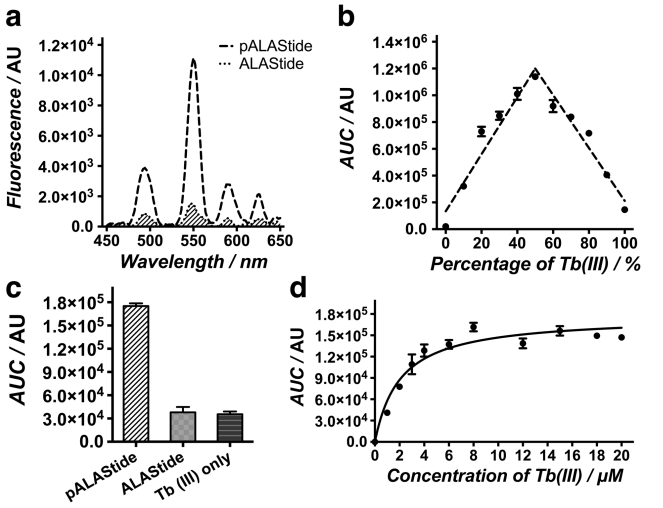
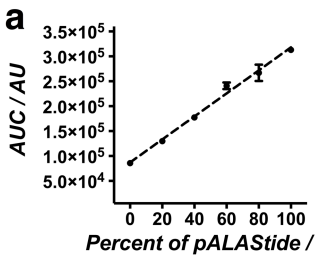


Figure 2. The binding behavior of pALAStide and Tb^{3+}. (a) ALAStide sensitizes Tb^{3+} luminescence upon its phosphorylation. (b) The Job's plot shows that pALAStide binds Tb^{3+} with 1:1 stoichiometry. (c) Unphosphorylated ALAStide does not sensitize Tb^{3+} luminescence. (d) One-site binding of pALAStide with Tb^{3+} is suggested, with dissociation constant $K_d = 0.8 \pm 0.2$ μM. Data indicate averages ± SEM from triplicate experiments. AUC: area under curve.

The binding of pALAStide and Tb^{3+} was further characterized in order to establish appropriate kinase assay conditions. The stoichiometry of pALAStide-Tb^{3+} binding was determined by the Job's method, in which the total concentration of pALAStide and Tb^{3+} were kept constant while their ratio varied. The Job's plot of the resulting time-resolved luminescence data showed that the highest luminescence intensity was reached when the concentration ratio of pALAStide and Tb^{3+} was equal to 1:1. Increasing or decreasing this ratio resulted in a linear decrease in luminescence intensity, indicating that the 1:1 binding ratio was the preferred stoichiometry in this binding process (Figure 2b).

Binding affinity of pALAStide with Tb^{3+} was also determined. In the presence of 2 μM pALAStide, samples with increasing Tb^{3+} concentration from 0 to 20 μM were prepared and their time-resolved luminescence spectra were collected. The binding curve was hyperbolic and reached saturation at 20 μM of Tb^{3+}, suggesting one-site binding behavior of the pALAStide (Figure 2d). The calculated K_d for this pALAStide-Tb^{3+} complex was 0.8 ± 0.2 μM, which is quite high Tb^{3+} affinity in the context of other known Tb^{3+} binding sequences[15,17,23].

We then demonstrated the quantitative detection of ALK activity and its inhibition by crizotinib using ALAStide. Prior to the kinase assay, a quantitative calibration curve was plotted using mixtures of ALAStide and pALAStide in the presence of components from kinase reaction buffer and quenching buffer (to best mimic kinase assay conditions) (Figure 3a). The calibration curve was linear with excellent reproducibility. The buffer components (particularly ATP) attenuated the signal to noise ratio compared to peptide/Tb^{3+}- only solutions (Supplementary Information), but assay parameters calculated from the calibration curve were still compatible with HTS requirements[24]: the calculated Z' factor and signal window (SW) values are given in Fig. 3a. (Z' > 0.5 and SW > 2 are generally required for HTS).



HTS parameters of calibration curve

P %	Z'	SW	CV
0%	N/A	N/A	0.040
20%	0.653	8.815	0.046
40%	0.851	29.475	0.027
60%	0.842	21.029	0.045
80%	0.748	10.219	0.087
100%	0.909	41.643	0.028

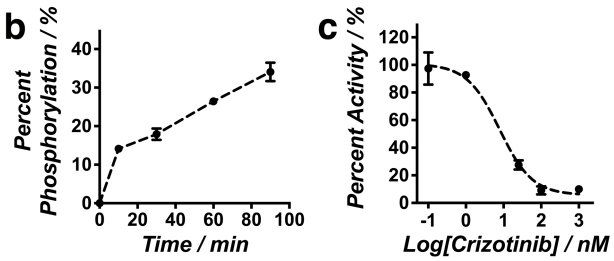


Figure 3. Detection of ALK activity and inhibition using ALAStide. (a) The calibration curve, established using the kinase assay buffers and conditions. The high-throughput screening (HTS) parameters calculated from the calibration curve are shown in the table. (b) Quantitative monitoring of ALAStide phosphorylation by ALK. (c) Dose-response inhibition of ALK by crizotinib. Data indicate averages ± SEM from triplicate experiments. P%, percent of phosphorylation. Z', Z' factor. SW, signal window. CV, coefficients of variation.

Commercial recombinant ALK kinase domain was used for assays on ALAStide. Briefly, aliquots of the reaction mixture were quenched at selected time points, luminescence buffer containing Tb^{3+} was added to the quenched sample, and time-resolved luminescence was measured. The area under the spectrum (automatically integrated by the instrument software)

was used as the metric for the assay, and the percentage of peptide phosphorylation was calculated according to the calibration curve (Figure 3b). Using the same procedure, we also examined the IC_{50} value of ALK inhibitor crizotinib in this assay. Crizotinib was diluted to final concentrations ranging from 0.1 nM to 1 mM. The total volume percentage of DMSO was limited to 0.1%, and each measurement was quantified by the calibration curve. The observed biochemical IC_{50} of crizotinib was 7.8 nM (Figure 3c), a reasonable value when compared with the previously reported IC_{50} value from a cell-based assay[7] (24 nM). This result demonstrated proof-of-concept and the promising potential of this assay, which could be further optimized and applied in actual ALK inhibitor screening in drug development practices.

In summary, we have developed a novel ALK biosensor, ALAStide, designed by a straightforward strategy that integrates lanthanide binding residues into a kinase substrate. ALAStide directly sensitizes Tb^{3+} luminescence upon its phosphorylation, enabling a quantitative and sensitive kinase assay for ALK. Utilization of time-resolved lanthanide luminescence provided substantially higher dynamic range and signal-to-noise ratio than typical biochemical assays, while no antibodies or chemical labels were used. Because this assay is essentially homogenous, requiring only reagent addition for the detection mode, this biosensor should be easily integrated into rapid, high-throughput screening of ALK inhibitors with corresponding optimization of the assay conditions. The ongoing development of novel inhibitors for ALK fusion kinases and their mutants should benefit from the streamlined workflow provided by this research. Moreover, by using information about known substrate preferences and adapting well-characterized, existing sequences in a way that minimizes the disruption of kinase specificity and phosphorylation efficiency, the same strategy can be easily applied to other kinases of drug development interest for cost-effective, high-throughput drug screening applications.

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Notes and references

[a] Department of Medicinal Chemistry and Molecular Pharmacology, College of Pharmacy, Purdue Center for Cancer Research, Purdue University, West Lafayette, IN 47907, United States

Current address: Department of Biochemistry, Molecular Biology and Biophysics, College of Biological Sciences, University of Minnesota, Minneapolis, MN 55455, United States.

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‡ Dr. Parker owns equity in and serves on the Scientific Advisory Board for KinaSense, LLC, a start-up from her laboratory which has licensed the intellectual property that includes the sensor reported in this work. This relationship has been reviewed and managed by both Purdue University and the University of Minnesota in accordance with their conflict of interest policies.

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