Communication

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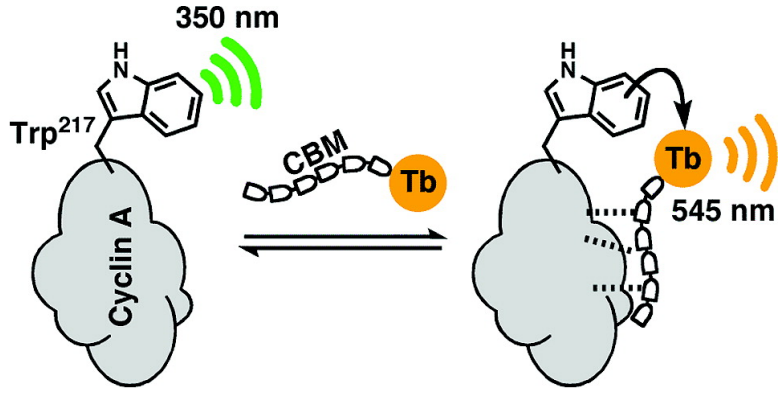

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Cyclin A Probes by Means of Intermolecular Sensitization of Terbium-Chelating Peptides

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Cell cycle progression is tightly regulated by the activity of several kinase complexes (kCDKs) formed by the association of cyclin-dependent kinases (CDK) and a regulatory cyclin unit.[1] Genetic evidence supports a strong correlation between alterations in the regulation of kCDKs and abnormal cell proliferation,[2] therefore these complexes represent attractive targets for the development of tumor suppression agents.[3] One of the most promising strategies for obtaining new kCDK inhibitors consists of targeting the cyclin A substrate recruitment element called the cyclin binding groove (CBG).[4,5] This task might greatly benefit from the existence of fluorescent probes that provide a signal upon specific binding to the groove. Moreover, such sensors might also be of unique utility to signal the presence of the protein in complex cellular environments. Herein we describe the design and synthesis of an efficient and selective cyclin A fluorescent sensor.

Lanthanide-based probes have received a great deal of attention in recent years owing to their unique spectroscopic properties.[6] In these applications, lanthanide ions are usually excited through intramolecular energy transfer from aromatic residues in their vicinity.[7,8] In contrast with the widespread application of such intramolecular sensitization, the use of *intermolecular* lanthanide sensitization processes is very scarce and essentially limited to the development of chemical sensors for aromatic molecules or ions.[9,10]

The CBG of cyclin A is partly defined by a Trp residue (217), which is involved in hydrophobic contacts with the binding partners through its indole side chain. We realized that such a Trp could be used as an antenna to sensitize a lanthanide ion incorporated at an appropriate position of a cyclin-binding peptide, no further than 15−20 Å from the Trp. The design of the peptides was based on the well-known consensus sequence for specific cyclin A binding (CBM, cyclin binding motif), Arg/Lys-Xaa-Leu, where Xaa is any amino acid.[11,4] Fine-tuning of this core model sequence on the basis of reported peptide library screenings[12] led us to design peptide **1**, which incorporates the Tb^{3+} chelating macrocycle DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) at the N-terminus of the optimized CBM.

Preliminary molecular modeling studies based on the X-ray structures of cyclin A bound to small peptides[13] suggested that the octapeptide **1** would place the metal center in relative proximity to the sensitizing aromatic Trp^{217} antenna,[14] while keeping intact the structural requirements for high-affinity binding and avoiding unwanted steric clashes with the protein surface (Figure 1). To study the influence of the metal ion location relative to the Trp antenna, we also synthesized peptide **2**, in which the chelating DOTA unit is attached to a nonessential Lys side chain in the peptide that is oriented away from the surface of the protein, and peptide **3**, featuring the DOTA attached to the shorter side chain of a 2,3-diamino propanoic acid (Dap) residue introduced in the same position. We also synthesized the control peptide **4**, which lacks the DOTA macrocycle (Scheme 1).

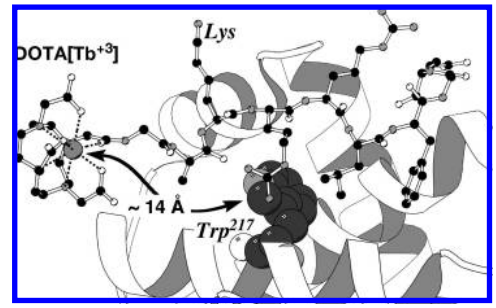
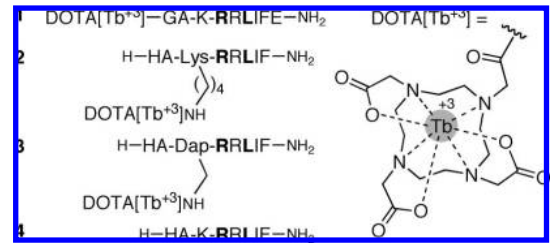


Figure 1. Proposed interaction of the terbium-chelating octapeptide **1**, with the binding groove of cyclin A based on the X-ray structure of cyclin A bound to a peptide inhibitor.[13] This interaction places the metal center near the Trp^{217} side chain (∼14 Å). The Lys residue modified in control peptides **2** and **3** is also labeled.

Scheme 1. Designed Terbium-Chelating Peptides[a]



[a] Key CBM residues are in bold. Dap = 2,3-diaminopropanoic acid residue.

Detailed synthetic procedures are given in the Supporting Information. In short, peptides were assembled following standard Fmoc solid phase peptide synthesis protocols,[15] and the DOTA unit was then introduced into the peptide scaffold as the tri'Bu-protected acid while the peptides were still attached to the solid support. For peptides **2** and **3** an orthogonal (alloc) protection scheme was used to allow selective modification of the Lys and Dap side chains, respectively. HPLC-purified peptides were subsequently complexed with $TbCl_3$ in HEPES buffer to give the desired chelates, which were purified again by HPLC.

Control fluorescence experiments confirmed that **1** was essentially nonfluorescent upon irradiation at 280 nm. Cyclin A displayed a strong Trp fluorescence emission band under these conditions, but the emission remained unchanged upon addition of up to 10 equiv of $TbCl_3$ or chelated terbium in the form of DOTA[Tb^{3+}] complex.

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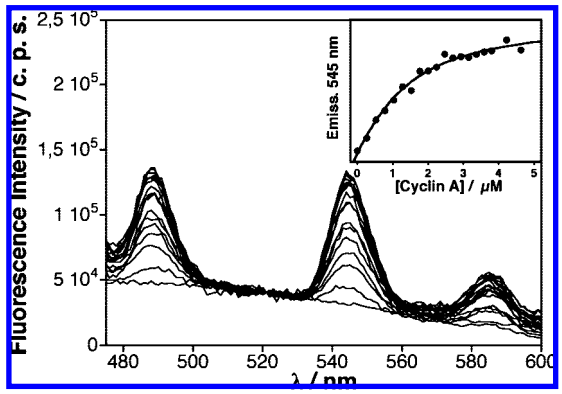

Figure 2. Fluorescence spectra of **1** (1 μM, 10 mM HEPES buffer, pH 7.5, 100 mM NaCl, $\lambda_{exc} = 280$ nm) with increasing amounts of cyclin A. The insert shows the plot of the fluorescence emission intensity at 545 nm with the best-fitting binding curve (see Supporting Information for curve fitting model and details).

In contrast, the addition of successive aliquots of a cyclin A solution (37 μM stock) to a 1 μM solution of peptide **1** resulted in the appearance of the three characteristic Tb^{3+} emission bands, with the most intense emission at 545 nm. This effect could only be observed when both cyclin A and **1** were simultaneously present in the solution. The increased emission at 545 nm followed a typical saturation profile that allowed the calculation of a binding constant of 895 $\pm$ 190 nM (Figure 2, insert).[16] Peptide **2**, which contains the DOTA ligand in the Lys side chain, also exhibited the typical lanthanide emission upon cyclin A addition, albeit with weaker intensity (Supporting Information). This suggests that in this complex the metal ion is located further from the Trp^{217} than in the case of **1**. Homologous peptide **3**, containing a much shorter linker between the peptide backbone and the DOTA, showed a very weak response to cyclin A. Modeling studies suggest that the Dap side chain is too short to allow efficient docking of peptide **3** in the CBG, thus preventing efficient recognition and energy transfer.

To verify our model we performed control experiments in which the bound peptide **1** was displaced with the competitive nonfluorescent peptide **4**. As expected for a competitive displacement, the Tb^{3+} emission decreased upon successive additions of the control until it eventually disappeared. The specificity of the fluorescent probe was further confirmed by measuring the emission of **1** in the presence of nonspecific control proteins, like *bovine serum albumin* (BSA), *immunoglobulin G* (IgG), or *myelin basic protein* (MBP), none of which was capable of sensitizing the metal ion (see Supporting Information).

To test the system in more demanding and biologically relevant conditions, we measured the emission of peptide **1** (1 μM) with increasing amounts of cyclin A in the presence of extracts from serum-starved cells that express negligible levels of cyclin A (total nonspecific protein concentration: 290 μg/mL). Peptide **1** showed extraordinary specificity as emission was not observed until cyclin A was added to the mixture. This is particularly relevant since more standard probes based on environment-sensitive fluorophores cannot usually display this level of selectivity. The total fluorescence emission intensity was slightly lower than in the experiments with purified cyclin A, with the detection limit being approximately 30 μg/mL.

In summary, we have shown that intermolecular sensitization of lanthanide ions offers a particularly useful strategy for the design of cyclin A biosensors. The signal increase is large and the emission takes place at long wavelengths, thereby making this approach suitable for biological studies. The methodology should be of general utility to obtain sensors for other biomolecular systems.

Current work is focused on refining the design to increase the affinity constant for cyclin A and hence improve the energy transfer efficiency in order to enable the detection of endogenous levels of cyclin in cell lysates. We are also studying the use of this approach to discover specific cyclin inhibitors by using competition assays.[17]

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Supporting Information Available: Synthetic and spectroscopic details and protein expression procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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