TrisNTA chelators consist of...

... three metal-chelating N-nitrilotriacetic acids (NTAs) connected by a scaffold structure. Coupled to a fluorophore or other reporter, trisNTAs can be used as a probe for live-cell labeling of histidine-tagged proteins. In their Communication on page 12395 ff., R. Tampé et al. report large differences in affinity and stability between linear, dendritic, and cyclic scaffolds, and clarify which trisNTA scaffold is superior for in vitro and cellular applications.
The Scaffold Design of Trivalent Chelator Heads Dictates Affinity and Stability for Labeling His-tagged Proteins in vitro and in Cells

And the winner is: The scaffold design controls the interaction of minimalistic trivalent NTA chelators for in vitro and cellular labeling of His-tagged proteins. A range of NTA-based multivalent chelator heads (MCHs) built on linear, cyclic, and dendritic scaffolds were compared and a cyclic trisNTA chelator was found to display the highest affinity and kinetic stability.

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Keywords: biosensors · His-tag · multivalence effects · protein labeling · target screening

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The Scaffold Design of Trivalent Chelator Heads Dictates Affinity and Stability for Labeling His-tagged Proteins in vitro and in Cells

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Abstract: Small chemical/biological interaction pairs are at the forefront in tracing protein function and interaction at high signal-to-background ratios in cellular pathways. However, the optimal design of scaffold, linker, and chelator head still deserve systematic investigation to achieve the highest affinity and kinetic stability for in vitro and especially cellular applications. We report on a library of N-nitrilotriacetic acid (NTA)-based multivalent chelator heads (MCHs) built on linear, cyclic, and dendritic scaffolds and compare these with regard to their binding affinity and stability for the labeling of cellular His-tagged proteins. Furthermore, we describe a new approach for tracing cellular target proteins at picomolar probe concentrations in cells. Finally, we outline fundamental differences between the MCH scaffolds and define a cyclic trisNTA chelator that displays the highest affinity and kinetic stability of all reported reversible, low-molecular-weight interaction pairs.

Decoding protein pathways and interactions is essential for building a system-based understanding of biological processes as well as their dysregulation in diseases.[1] Generic tools are eagerly awaited to trace proteins by using small chemical probes at high spatiotemporal resolution.[2] Protein labeling can be realized by chemical modification and or enzyme- or affinity-mediated attachment of certain precursors.[3] These methods offer versatile ways to analyze the activity, conformational states, cellular localization, and interactions of the target protein.

Metal-chelating N-nitrilotriacetic acid (NTA) and the oligohistidine tag (His-tag) constitute a well-established interaction pair, which was first described in immobilized metal-ion affinity chromatography (IMAC).[4] Specific binding is based on the formation of an octahedral complex of bivalent metal ions, such as Ni²⁺ or Co²⁺, with histidines (Figure 1c). The interaction is reversible and can be released by competition with histidine or imidazole. So far, the HisTag is the smallest and most commonly used affinity tag in life sciences, thus making it an attractive target for site-specific protein labeling and in vitro or in vivo screening approaches. Initial attempts to His-tag labeling utilized Ni-NTA-modified dyes.[5] However, the low affinity ($K_d \approx 10 \mu M$) and the fast off-rate ($k_d \approx 1 \text{s}^{-1}$) resulted in a very transient labeling by the His-tag/Ni-NTA pair.[6,7] To overcome this limitation, we introduced the concept of multivalent chelator heads (MCHs) by exploiting a molecular avidity effect. The MCHs contain two to six NTA units, leading to (sub-)nanomolar binding affinity towards a histidine tag for site-specific labeling of targets even in a crowded cellular environment with low toxicity.[6,7] The combination of high-affinity binding properties and an ultra-small, non-disturbing affinity tag of smaller than 1 kDa enables 1) protein labeling with organic dyes,[9] quantum dots[9] and streptavidin,[10] 2) protein tethering to membranes,[11] liposomes,[12] glass surfaces,[13] gold interfaces,[14,15] and nanoparticles,[15] 3) guided high-affinity protein trans-splicing and “traceless” labeling,[14] and 4) stochastic sensing of single molecules in nanopores,[17] single-molecule tracking,[18] or super-resolution microscopy even in living cells.[16,17] However, the variety of arrangements for connecting the NTA groups has led to a confusing array of MCH probes on offer, sometimes even sharing the same designation, despite a fundamentally different chemical structure.

Herein, we disclose substantial differences between cyclic, linear, and dendritic MCHs, thus underlining that the structural organization of the NTA elements is important for high affinity and kinetic stability. Furthermore, we describe the fine-tuned optimization of trivalent chelator heads towards the highest affinity (i.e., lower probe concentration) and kinetic stability, as well as specificity (lower background). As a final milestone, we determined which of all currently available MCH probes are optimal for life science applications.

We focused on the flexibility of the NTA-linker arm and the rigidity of the cyclic scaffold. For that purpose, new cyclic cyclam-Asp-trisNTA (2) and cyclen-Glu-trisNTA (3) were compared with cyclam-Glu-trisNTA (1), linear trisNTAs 4 and 5, and dendritic NTA-Dab-trisNTA (6) and NTA-Orn-trisNTA (7); Figure 1a). The synthesis of cyclam-Asp-trisNTA (2) and cyclen-Glu-trisNTA (3) is related to that of cyclam-Glu-trisNTA (1)[6] (Figure S1 in the Supporting Information). NTA-Orn-trisNTA (7) was synthesized as previously described,[20] while NTA-Dab-trisNTA (6) was obtained through a new synthesis route (Figure S2). Linear trisNTAs 4 and 5 were synthesized as recently described[21] (Figure S3). To compare their kinetic and thermodynamic parameters ($k_a$, $k_d$, $K_d$), all trivalent chelator heads were conjugated to biotin via a flexible spacer to allow highly controlled immobilization for interaction analysis with His-tagged proteins using surface plasmon resonance (SPR). The MCHs were immobilized at the lowest possible surface density to exclude any bias arising from a multivalency effect at the sensor interface. This
An important aspect is often neglected when multivalent compounds are analyzed at interfaces. Based on the maximum binding capacity of the neutravidin-functionalized SPR chip [300 resonance units (RU) for cyclam-Glu-trisNTA (1)], we ensured a very low immobilization density of 10 RU for all biotin-PEG<sub>4</sub>-trisNTA compounds. Utilizing the His<sub>6</sub>-tagged maltose-binding protein (MBP-H<sub>6</sub>), the kinetic and thermodynamic binding properties of all MCHs were compared by SPR spectroscopy (Figure 1b–d), resulting in binding affinities in the range of 10–60 nm. All data were corrected for unspecific binding and were fitted to a Langmuir 1:1 isotherm.<sup>[22]</sup>

The respective kinetic parameters are summarized in Table 1. A full dataset is given in Table S2. Interestingly, the
Table 1: Kinetic and thermodynamic parameters of Ni\(^{3+}\)-loaded MCHs coordinating His\(_{10}\)-tagged MBP analyzed by SPR spectroscopy.

<table>
<thead>
<tr>
<th>trivalent NTA</th>
<th>Cyclic</th>
<th>Linear</th>
<th>Dendritic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>(k_a [10^5 \text{ M}^{-1} \text{s}^{-1}])</td>
<td>3.21 ± 0.01</td>
<td>5.17 ± 0.04</td>
<td>2.49 ± 0.01</td>
</tr>
<tr>
<td>(k_d [10^{-5} \text{s}^{-1}])</td>
<td>3.05 ± 0.01</td>
<td>27.40 ± 0.05</td>
<td>5.97 ± 0.02</td>
</tr>
<tr>
<td>(K_D [\text{nm}])</td>
<td>9.51 ± 0.06</td>
<td>53.10 ± 0.51</td>
<td>24.00 ± 0.18</td>
</tr>
</tbody>
</table>

The cyclam-Glu-trisNTA system (1) appears to be optimized with regard to binding affinity and stability. A further option to increase the binding affinity would be the expansion of the His-tag (e.g., ten or twelve consecutive histidines). For MBP-H\(_{10}\) and MBP-H\(_{12}\), the \(K_D\) values obtained by SPR spectroscopy ranged from 0.5 to 4.0 nm (Figure S5 and S6). Notably, SPR analyses with very slow dissociation rates are difficult. Therefore, the MCH/His-tag interaction was challenged by increasing concentrations of imidazole (10 nm and 30 nm), which displaces the histidines and hence weakens the MCH/His-tag interaction. The complex stability with MBP-H\(_{10}\) was strongly increased for cyclam-Glu-trisNTA (1) and cyclen-Glu-trisNTA (3), thus highlighting the important role of the cyclic scaffold. For comparison, the dissociation phase of MBP-H\(_{12}\) was normalized (Figure 2a–c) and the dissociation rates were displayed as half-life times (Figure 2d; see Figure S9 for full dataset; see Figure S7 and S8 for full dataset for MBP-H\(_{10}\) and MBP-H\(_{12}\)).

In terms of the entropic freedom of the cyclic scaffold, we concluded that the differences between the cyclam (flexible) and the cyclen (more rigid) scaffold only have a minor influence on the binding stability. In contrast, the NTA linker arm has a strong impact as illustrated by the comparison of 1, 2, and 3. The long glutamate-based NTA linker arm in 1 and 3 is crucial for a stable interaction. These results confirm the large difference in the kinetic stability of the diverse trivalent chelator compounds. In conclusion, the trivalent cyclam chelator heads cyclam- and cyclen-Glu-trisNTA (1 and 3) displayed significantly higher kinetic stability compared to the linear or dendritic trisNTA, which is also reflected in the SPR data. Taking the results of all trivalent chelator heads into account, the cyclic scaffold as well as the linker of cyclam-Glu-trisNTA (1) is optimized for high affinity and stable binding of His-tagged proteins.

To further clarify the observed differences in affinity and kinetic stability, we compared cyclic and linear trisNTA fluorophore conjugates as probes for intracellular protein labeling. To guarantee highest affinity, we additionally developed a novel MCH labeling protocol in fixed and permeabilized cells. Based on this finding that consecutive histidines are prone to chemical modification during chemical fixation, we examined the direct labeling of intracellular His-tagged proteins by MCH fluorescent probes using glyoxal.[23]

For comparison, we chemically arrested mammalian cells using conventional paraformaldehyde (PFA)[24] or glyoxal and imaged labeling using cyclic trisNTA coupled to the bright fluorophore Alexa647 (40) by confocal laser-scanning microscopy (CLSM). HeLa Kyoto cells transiently expressing His\(_{10}\)-mEGFP-LaminA, which is located at the nuclear envelope, revealed a strong and precise labeling at picomolar probe concentrations after glyoxal treatment, while PFA-treated cells showed no significant labeling under similar conditions (Figure 3a/b). We therefore hypothesized that the accessibility of the His-tag is substantially improved using our advanced MCH labeling method. For a final statistical evaluation of the MCH scaffolds, we added 100 pM of either cyclic trisNTA\(^{cys}\) (18) or linear trisNTA\(^{cys}\) (34) probes to glyoxal-fixed HeLa Kyoto cells transiently expressing His\(_{10}\)-mEGFP-LaminA. We mimicked SPR conditions and a live-cell environment by subsequently adding 10 mM imidazole (Figure S10). Afterwards, we imaged the cells by CLSM and analyzed the ratio of bound probe relative to the His\(_{10}\)-mEGFP-LaminA by densitom-
etry (Figure 3c). Strikingly, we observed approximately 3–4 times more cyclic trisNTA<sup>des</sup> (18) bound to His<sup>10-mEGFP</sup>LaminA in contrast to the linear trisNTA<sup>des</sup> (34). We further delivered the cyclic trisNTA probe into living cells and observed highly specific labeling of His<sup>10-mEGFP</sup>LaminA (Figure S11).

In conclusion, we systematically investigated the importance of the scaffold design of trivalent MCHs with regard to affinity and kinetic stability. Corresponding fundamental distinctions were visualized by a comprehensive SPR study of MCHs differing in their basic structural arrangement: linear, cyclic, dendritic. Furthermore, we directly contrasted cyclic trisNTA and linear trisNTA fluorophore conjugates for the tracing of intracellular His-tagged proteins. We also developed a new glyoxal-fixation method for MCH labeling, which enabled the use of picomolar probe concentrations. Ultimately, we identified cyclic trisNTA as superior for in vitro and cellular applications compared to the other described trivalent chelator heads. Thus, we suggest a detailed nomenclature for MCH molecules or to henceforth limit the simple term trisNTA to cyclam-Glu-trisNTA (cyclic trisNTA) in order to avoid confusion. We hypothesize that, in combination with established live-cell delivery techniques for MCH probes, like cell-squeezing or genetically encoded nanopores, these structural details and optimized trivalent MCHs could potentially enable chemically induced dimerization. Finally, these nanotools will open up new perspectives for ultra-small, non-disturbing, and high-affinity labeling to gain deeper insights into dynamic cellular structures and processes.

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Conflict of interest

The authors declare no conflict of interest.

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