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Mass Spectrometric Screening of Ligands with Lower Off-Rate from a Clicked-Based Pooled Library

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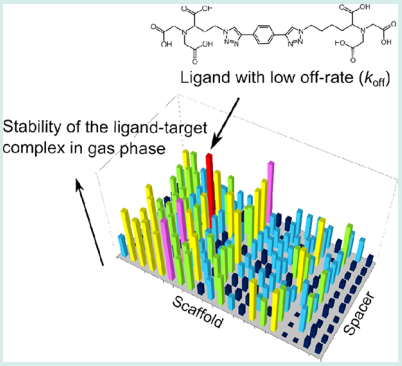
Supporting Information

ABSTRACT: This paper describes a convenient screening method using ion trap electrospray ionization mass spectrometry to classify ligands to a target molecule in terms of kinetic parameters. We demonstrate this method in the screening of ligands to a hexahistidine tag from a pooled library synthesized by click chemistry. The ion trap mass spectrometry analysis revealed that higher stabilities of ligand-target complexes in the gas phase were related to lower dissociation rate constants, i.e., off-rates in solution. Finally, we prepared a fluorescent probe utilizing the ligand with lowest off-rate and succeeded in performing single molecule observations of hexahistidine-tagged myosin V walking on actin filaments.



KEYWORDS: *click chemistry, fluorescent probes, high-throughput screening, mass spectrometry*

Various methods to screen chemical libraries with huge diversity have been developed in the fields of catalysis, material science and medicinal chemistry.[1] Recently, electrospray ionization mass spectrometry (ESI-MS) has attracted attention as a potential tool for label-free screening in a high-throughput format.[2] This strategy has already been implemented in catalyst screening through the investigation of reaction intermediate species.[3] Such an approach allows a readout of ligand-target noncovalent interactions from a pooled library without the need to purify each ligand.[4] We have also investigated the relationship between the stability of the ligand-target noncovalent complex in gas phase and the dissociation constant (K_D) in solution by using a collision activated dissociation method based on ion trap ESI-MS.[5] However, the relationship between the stability in gas phase and kinetic parameters (k_{on} and k_{off}) in solution remains ambiguous.

In this paper, we examine this relationship through the screening of ligands which favor a hexahistidine tag (His-tag) as a target molecule.[6] The best screened ligand was then applied to the creation of a new fluorescent probe to label His-tagged proteins. His-tag ligands are widely used in affinity chromatography to purify His-tagged proteins. Nitrilotriacetic acid (NTA), the most common such moiety, is capable of recognizing two imidazolyl rings of histidine units with one nickel ion complexed by the ligand. To develop ligands with strong affinity for a His-tag, scaffolds bearing two or three NTA moieties, so-called bis- or tris-NTA ligands, have been studied.[7] By estimating the structure of hexahistidine, those researchers designed and synthesized several multiple NTA ligands. However, the three-dimensional structure of the His-tag peptide in solution is difficult to determine due to the flexibility of the peptide.[8] Hence, it is not easy to computationally design the scaffold and spacers required for multiple NTA ligands to achieve optimum binding to the target. In contrast, very few reports have appeared describing the systematic screening of multiple NTA ligands, even bis-NTA.[7]

A pooled library containing various bis-NTA ligands in solution was prepared based on the azide−alkyne Huisgen cycloaddition click reaction.[9] NTA derivatives bearing an azide group on the end of four different aliphatic chain lengths (1−4 methylene units) were combined with a selection of 20 bis-terminal alkyne spacer groups to generate a total of 200 bis-NTA ligands with substantial structural diversity (Figure 1).

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The bis-alkyne scaffolds included functional groups such as five- or six-membered aromatic rings (structures **a**–**j**) and aliphatic chains (**k**–**t**).

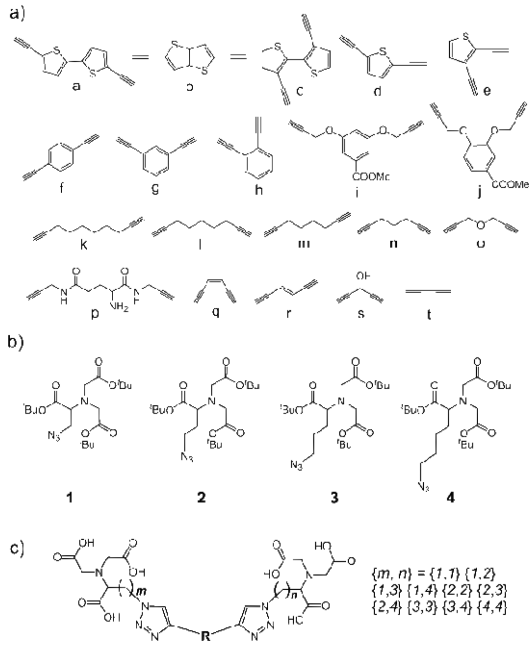

Figure 1. Synthesis of 200 compounds for the chemical library by click chemistry. (a) To synthesize branched structures, bis (alkynes) from **a** to **t** were used as clickable scaffolds. (b) As a recognition moiety, nitrilotriacetic acid (NTA) with different carbon numbers having an azide group at the terminus were synthesized as a *tert*-butyl ester protected form. Each NTA with an azide group was termed as **1**, **2**, **3** and **4**. (c) The "Click" conjugation of one bis (alkynes) scaffold to four different azide NTAs generated ten species. The nomenclature R is from **a** to **t** and bis-NTA ligands were named as follows; (**alphabet**){*m*, *n*}.

The bis-terminal alkynes were commercially available or synthesized by a simple Sonogashira reaction (see the Supporting Information).[10] As a recognition moiety, the four azidoalkyl-functionalized NTA components (**1**–**4**, Figure 1b) were synthesized in *tert*-butyl protected form (see the Supporting Information). A typical ESI-MS sample was prepared as follows: one scaffold (**t**) was reacted with an equimolar mixture of **1**, **2**, and **4** in a single flask in the presence of copper sulfate and sodium ascorbate in THF/water(v/*v* = 1/1) at room temperature. After the products were separated from the catalyst by extraction with CH_2Cl_2, *tert*-butyl groups were deprotected with TFA. The resulting library contained a total of six compounds, both symmetric and unsymmetric: **t**{*1,1*}, **t**{*1,2*}, **t**{*1,4*}, **t**{*2,2*}, **t**{*2,4*}, and **t**{*4,4*}. Because of the reliability of the click reaction and its insensitivity to the steric nature of the azide group, we expected these candidate ligands to be formed in the statistical ratio of 1:2:2:1:2:1. The relative intensities observed by ESI-MS supported this assumption (see Supporting Information). The same result was observed for each of the other scaffolds, indicating that the reactivity was independent of the scaffold used. A major advantage of the click reaction approach is that a simple extraction can be used to separate the catalyst components from the library, which may otherwise interfere with the ionization process. Library mixtures were designed so that different structures with the same molecular weight, such as {*1,3*}–{*2,2*}, {*1,4*}–{*2,3*}, and {*2,4*}–{*3,3*}, were not produced in the same solutions (see the Supporting Information). This strategy avoids the generation of complexes that cannot be distinguished by ESI-MS.

Each mixture of the library was screened by the collision activated dissociation method in an ion trap ESI-MS instrument. The bis-NTA ligands were mixed with $NiCl_2$ in a 1:1 molar ratio (Ni:NTA), and the complexes were treated with hexahistidine (**His**). The resulting noncovalent ternary complexes were detected as divalent negative ions in ESI-MS. For example, when **His** was added to the library pools containing **t**{*1,1*}, **t**{*1,2*}, **t**{*1,4*}, **t**{*2,2*}, **t**{*2,4*}, and **t**{*4,4*}, six species, all ternary complexes with **His** and two nickel ions, were detected in a single scan as shown in Figure 2a.

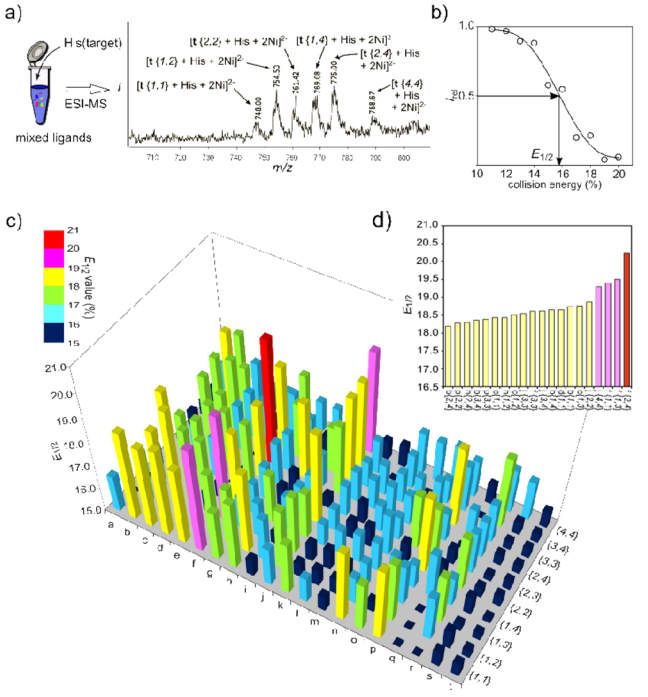

Figure 2. Screening of the mixed ligands library by ion trap ESI-MS. (a) Hexahistidine (**His**) as target was added to the solution containing various bis-NTA ligands (for example, **t**{*1,1*}, **t**{*1,2*}, **t**{*2,2*}, **t**{*1,4*}, **t**{*2,4*}, and **t**{*4,4*}) preloaded with nickel ions (Ni^{2+}), to form the ligand-target complex. ESI-MS detects peaks corresponding to the ternary complex with His and each bis-NTA ligand-Ni × 2 as double negative ion charge. (b) Each complex is isolated within the ion trap and the stability of the complex is evaluated by collision activated dissociation. $E_{1/2}$ is defined as the index of the stability, where the relative abundance of the complex (I_{rel}) reaches 0.5. (c) $E_{1/2}$ from 200 bis-NTA ligands were divided into six categories (i.e., <16%, 16–17%, 17–18%, 18–19%, 19–20% and >20%), which were color coded. (d) Top 20 bis-NTA ligands from among the 200 library members.

Complexes with other ratios of His to bis-NTA ligand were not observed. Subsequently, each of the six desired ternary complexes species was isolated in the ion trap and subjected to collision with helium gas at increasing energies. Each complex was observed to dissociate into the ligand and target, such as [**t**{*1,1*}+Ni]$^-$ and [**His**+Ni]$^-$, without any problematic decomposition of the compounds. The decrease of the relative abundance of the complex as a function of collision energy was displayed as a collision profile (Figure 2b).

An index of the stability of ligand-target complexes, $E_{1/2}$, as described by Gross et al., was defined as the relative energy

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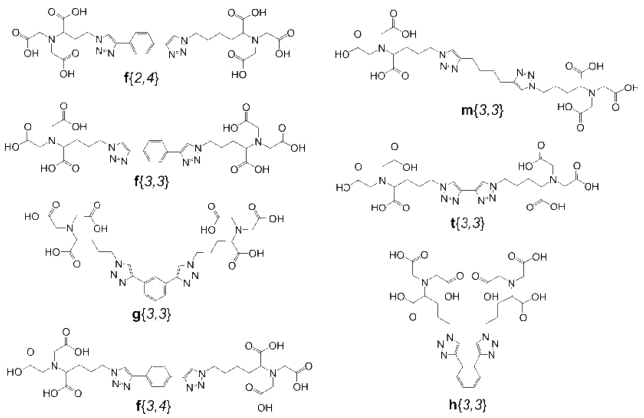
value at which the abundance of the complex reached 0.5.[11] These $E_{1/2}$ values represent the relative stabilities of the complexes in the gas phase, and show substantial differences in magnitude among the 200 bis-NTA compounds in the library (Figure 2c and Table S1 in Supporting Information). The rigid para-phenylene dialkyne **f** gave rise to several of the most stable structures, including the one displaying the highest $E_{1/2}$ value (**f**{*2,4*}). None of the flexible dialkynes of approximately the same alkyne−alkyne distance (**m**, **n**, **o**), nor the regioisomeric dialkynylbenzenes **g** and **h**, performed as well. Interestingly, the relatively flexible dialkyne **j** did provide several good ligands with the longer NTA-azides ({*3,3*}, {*3,4*}, and {*4,4*}). In several cases, single carbon differences in spacer length of the azide NTA changed the $E_{1/2}$ substantially even for the same scaffold (c.f. **f**{*2,4*} vs **f**{*3,4*} and **f**{*3,3*} vs **f**{*3,4*}), showing that overall affinities for histidine are a sensitive combination of multiple structural and dynamic factors.

We expected that a greater $E_{1/2}$ value would reflect a higher stability of the ligand-target complex in solution, that is, better ligand binding to **His** and hexahistidine tags. Previous work has address the question of whether ligand-target affinities in the gas phase measured with ESI-MS can be correlated with solution phase properties.[2,4] While such correlations have not been reliable for noncovalent complexes formed principally via hydrophobic interactions,[12] good agreement has been found in many cases of noncovalent complexes which are easily ionized by virtue of being charged.[13] Among these are noncovalent complexes formed via hydrogen bonding, coordination bonding, and electrostatic interactions.[5,13]

For this purpose, we individually synthesized several bis-NTA ligands covering a broad range of $E_{1/2}$ values: **f**{*2,4*}, **f**{*3,3*}, **f**{*3,4*}, **g**{*3,3*}, **h**{*3,3*}, **m**{*3,3*}, and **t**{*3,3*}. The kinetic parameters of each bis-NTA ligand in solution were determined by Biacore measurement of the interaction of the candidate bis-NTA species with biotinylated-hexahistidine immobilized onto an avidin-conjugated substrate (Table 1). Figure 3 shows good agreement between relative k_{off} and $E_{1/2}$ values: the higher the stability of a histidine complex in the gas phase, the lower the kinetic dissociation rate of the corresponding hexahistidine complex in the solution phase. On the other hand, k_{on} did not correlate with $E_{1/2}$ (Figure 3), presumably because the ion trap experiment does not probe the complex formation step. The correlation of $E_{1/2}$ with K_D was therefore not as strong as with off-rate. This observation indicates that care must be taken when analyzing the stability of a noncovalent complex in the gas phase evaluated by collision activated dissociation because the results do not always reflect the overall dissociation constant K_D.

The ligand possessing the slowest off-rate, **f**{*2,4*}, was fabricated into a fluorescent probe to target a His-tagged protein. Biotin-modified **f**{*2,4*} (Figure 4a) was synthesized and its binding parameters to His-tag determined as above (see Supporting Information). The resulting k_{off} value was 1.00×10^{-3} s^{-1}, slightly lower than that of the parent **f**{*2,4*} (1.31×10^{-3} s^{-1}). The biotinylated ligand was then immobilized onto a straptavidin-coated quantum dot. The resulting fluorescent probe was tested in a single-molecule myosin V movement assay because off-rate is a crucial factor for labeling his-tagged proteins under dilute conditions. Myosin V is a dimeric motor protein that transports organelles along actin filaments in cells.[14] Myosin V with a His-tag at the N-terminus was treated with the **f**{*2,4*}-based probe and observed under an optical microscope in the presence of ATP. As shown in Figure 4b, we

Table 1. Kinetic parameters derived from Biacore measurements



entry	$E_{1/2}$ (%)	k_{on} (10^3 $M^{-1}s^{-1}$)	k_{off} (10^3 s^{-1})	K_D (μM)[a]
f{*2,4*}	20.2	2.11 ± 0.03	1.31 ± 0.03	0.62 ± 0.03
f{*3,3*}	18.6	2.99 ± 0.04	1.48 ± 0.02	0.49 ± 0.01
g{*3,3*}	17.4	4.80 ± 0.06	2.29 ± 0.02	0.48 ± 0.01
f{*3,4*}	16.9	1.39 ± 0.11	3.58 ± 0.23	2.57 ± 0.37
m{*3,3*}	16.3	1.61 ± 0.03	7.73 ± 0.05	4.79 ± 0.12
h{*3,3*}	16.0	4.59 ± 0.11	6.76 ± 0.07	1.47 ± 0.05
t{*3,3*}	15.4	2.24 ± 0.06	8.10 ± 0.45	3.62 ± 0.31

[a] $K_D = k_{off}/k_{on}$.

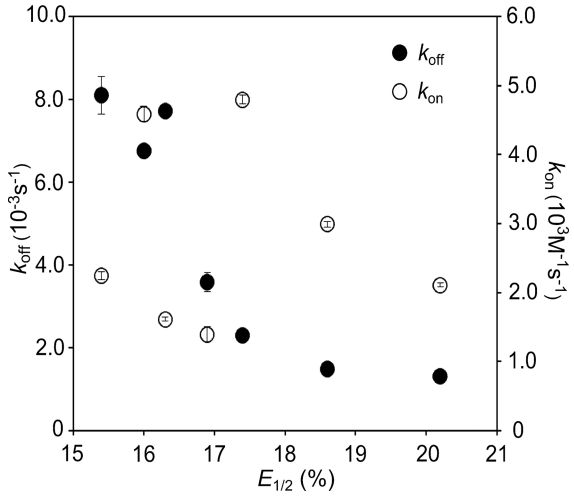


Figure 3. The relationship between $E_{1/2}$ and the kinetic parameters (k_{on} and k_{off}). The kinetic parameters were determined using a Biacore X-100. **f**{*2,4*}(the highest $E_{1/2}$), **f**{*3,3*}, **g**{*3,3*}, **f**{*3,4*}, **m**{*3,3*}, **h**{*3,3*} and **t**{*3,3*}, were plotted from right to left in decreasing order of the $E_{1/2}$ values.

successfully observed single Q-dots moving unidirectionally along an actin filament (see Movie S1 in the Supporting Information). The average velocity (453 nm s^{-1}, n = 72) and the average run length (741 nm, n = 72) are comparable to previous observations (Figure 4c and 4d).[15] These single-molecule observations, which are usually performed at low concentrations of protein (i.e., 1−10 nM), indicate that **f**{*2,4*} possesses a k_{off} value that is low enough to label the active motor protein.

In summary, the mass spectroscopic screening method described in this paper identified an asymmetric ligand with high affinity for a hexahistidine tag from a pooled library of mixed ligands synthesized by click chemistry. The isolation and

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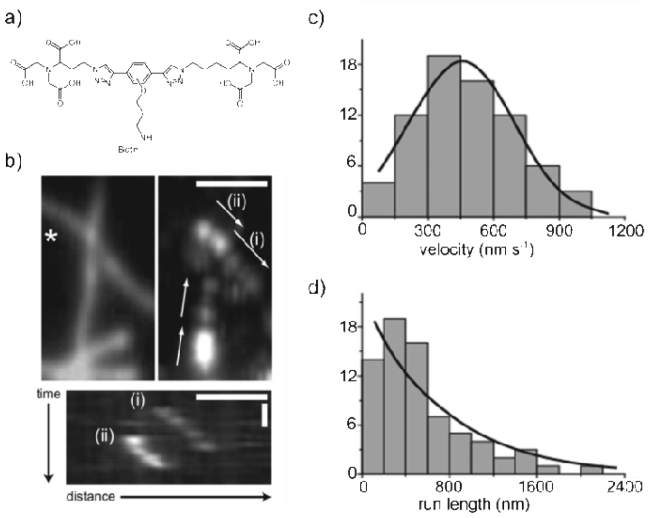

Figure 4. Observation of single myosin V molecules. (a) Biotin-modified **f**{2,4} (b) Top left: actin filaments. Top right: Q-dots moving along the actin filaments. Video frames were integrated for six seconds. Four moving Q-dots appeared as traces (white arrows). Bottom panel: kymograph along an actin filament in the top panel (*). Horizontal scale bars, 2 μm. Vertical scale bar, 1 sec. Brightness and contrast of images have been artificially enhanced. (c) Distribution of the running velocity. The thick line shows the fit to a Gaussian peaked at 453 nm s^{-1}. (d) Distribution of the run length. The thick line shows the fit to a single exponential, $p(x) = 21 \exp(-x/741)$, where $p(x)$ is the probability of myosin V traveling the distance x.

testing of candidate species in the ion trap mass spectrometer allows the evaluation of complex mixtures of asymmetric ligands without having to synthesize the individual species. The gas-phase stabilities of a set of complexes agreed with their dissociation rate constants in solution. The mass spectrometry screening method also has the capability to significantly improve drug development because off-rate is generally considered an effective parameter for predicting the pharmacological effect.[16] Furthermore, this is a particularly suitable tool in cases where the structure of the peptide or protein of interest is unpredictable. In order to make this method generic, other combinations of proteins and small molecules that rely on nonmetal interactions and have broad affinity ranges will be selected and examined in the future.[13]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures; synthesis of compounds, the characterizations, the detailed ESI-MS results and the microscope observation (supporting movies). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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