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Tris-Nitrilotriacetic Acids of Sub-nanomolar Affinity Toward Hexahistidine Tagged Molecules

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Abstract

Nitrilotriacetic acid (NTA) has moderate affinity (10 μ M) for hexahistidine (His6) and is widely used in the purification of His6-tagged proteins. The affinity can be increased significantly (10 nM) through multivalency such as using a tris-NTA. We show that the binding affinity of tris-NTA is dependent on the flexibility and length of the spacer between the mono-NTA and the scaffold: the shorter the spacer, the higher the affinity. A series of biotinylated tris-NTA having different spacers were synthesized and used to prepare tris-NTA sensor chips for surface plasmon resonance measurement of binding affinity. Sub-nanomolar affinity can be achieved with a short spacer. The new high affinity tris-NTA enables the formation of stable complexes with hexahistidine containing molecules and provides a convenient method to non-covalently attach proteins to various surfaces.

INTRODUCTION

Nitrilotriacetic acid (NTA) and its derivatives have broad applications in the manipulation of hexahistidine (His6) tagged proteins such as in immobilized metal affinity chromatography (1–2), crystallization on lipid template (3), fluorescent labeling (4–7) and surface immobilization (8). The advantages of the NTA-His6 interaction are reversibility and selectivity. However, the relatively weak affinity (equilibrium dissociation constant $K_D \sim 10 \mu$ M) between mono-NTA and His6 makes such technology unsuitable for applications where high affinity is required. Recent studies from Lata (9), van Broekhoven (10), and Huang (11) have demonstrated that a significant increase of binding affinity of NTA with His6-tagged proteins can be achieved through multivalency. For example, tris-NTA has a K_D around 10 nM toward His6-tagged proteins. Furthermore, the development of multifunctional tris-NTA provides a useful toolbox for the analysis and modification of His6-tagged proteins.

An important issue that has not been addressed is the influence of tris-NTA structure on the binding affinity. It remains a challenge to accurately predict the best scaffold and linker for a multivalent molecule (12). In the synthesis of tris-NTA, Lata used a tetraaza cyclam scaffold and glutamic acid spacer resulting in a K_D of 20 nM (9). We employed a dendritic lysine scaffold and a lysine spacer leading to a similar K_D (5 ~ 20 nM depending on the structure of His6-tagged protein) (11). Although the current generation of tris-NTAs exhibit high

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affinity, the interaction between the tris-NTA and His6 may not be strong enough to keep the conjugate intact in the biological environment, especially at a low concentration. Thus, it would be of interest to determine if higher affinity multi-NTA molecules can be obtained by changing the spacer between the scaffold and the mono-NTA (Figure 1). Here we report the structure-affinity relationship on modified tris-NTA and the realization of sub-nanomolar affinity with shorter spacers.

EXPERIMENTAL PROCEDURES

Materials and methods

H-Lys(Z)-OtBu.HCl and H-Orn(Z)-OtBu.HCl were from BACHEM (Torrance, CA). N-CBZ-amido-dPEG4-acid was from QuantaBioDesign (Powell, OH). Other reagents were from Aldrich (Milwaukee, WI). ¹H NMR (400 MHz) spectra were recorded on a Varian 400 MHz instrument. Chemical shifts are expressed as parts per million using tetramethylsilane as internal standard. *J* values are in Hertz. MALDI-TOF mass spectra were obtained at the Mass Spectrometry Facility, University of California, San Francisco. TLC analyses were performed on 0.25-mm silica gel F₂₅₄ plates. High performance flash chromatography (HPFC) was carried out on a Biotage (Charlottesville, VA) Horizon™ system with pre-packed silica gel columns (60 Å, 40–63 μm). Unless noted otherwise, the ratios describing the composition of solvent mixtures represent relative volumes.

General chemistry

Biotin-tris-NTAs (**6a–e**) were synthesized according to the previously published method for **6b** (11). The general synthetic route was outlined in Scheme 1. Various spacers were introduced in tert-butyl protected amino-mono-NTA (**2a–e**) by using different amino acids as the starting material. Compound **2b–c** were synthesized by using commercially available H-Lys(Z)-OtBu.HCl and H-Orn(Z)-OtBu.HCl, respectively. For the synthesis of compound **2d–e**, 2,4-diaminobutyric acid and 2,3-diaminopropionic acid were selectively protected by the carbobenzyloxy (Z) group and tert-butyl group first, then converted to amino-mono-NTA by the same method as **2b** (Scheme 2). Compound **2a** was obtained by the coupling of N-CBZ-amido-dPEG4-acid to **2b** followed by the removal of Z group (Scheme 3).

2-amino-3-(benzyloxycarbonylamino)propanoic acid (8e)—(13) 2,3-diaminopropanoic acid (10 g, 71.1 mmol) was dissolved in 150 mL pH 7 phosphate buffer. The pH of the solution dropped to 5.5 initially, and was adjusted to 6.8 with 1 M sodium hydroxide. A solution of benzyl chloroformate (10.2 mL, 71.1 mmol) in 10 mL toluene was added dropwise into the reaction mixture with vigorous stirring at 0 °C over 80 minutes. The pH of the mixture was monitored with a pH meter and maintained between 6.7 and 7.0 by addition of 1 M sodium hydroxide. The mixture was kept at 0 °C for another 4 h after the completion of addition, then 8 h reaction at room temperature. The white precipitate was collected by filtration, washed with water and ether consecutively. The white solid was recrystallized from water. Yield: 12.8 g, 71%.

tert-butyl 2-amino-3-(benzyloxycarbonylamino)propanoate (9e)—(14) Compound **8e** (5 g, 21 mmol) was suspended in anhydrous dioxane (40 mL) in a 150 mL pressure vessel. After the addition of 4 mL conc. H₂SO₄, the suspension turned clear. Isobutene (ca. 50 mL, cooled by dry ice-acetone bath) was poured into the solution, and the vessel was sealed. After 16 h reaction at room temperature, the cap of the vessel was carefully loosened and the pressure was released slowly. The reaction mixture was poured into a mixture of cold ether and 1 M NaOH (150 mL/210 mL) in a separation funnel, and shaken well. The ether layer was collected. The aqueous layer was washed with 100 mL cold ether. The ether layers were combined and dried over anhydrous sodium sulfate, evaporated under reduced

pressure, and placed under high vacuum. Yield: 5.0 g. TLC: R_f = 0.52 in CHCl_3 -MeOH (6:1). ^1H NMR (CDCl_3), δ 1.46 (s, 9H); 3.29 (m, 2H); 3.48 (m, 1H); 5.08 (s, 2H); 5.92 (br, 1H); 7.33 (m, 5H).

Compound 10e—Compound **10e** was synthesized according to the method of **10b** (**11**). ^1H NMR (CDCl_3), δ 1.45 (s, 9H); 1.47 (s, 18H); 3.08 (m, 1H); 3.40 (m, 1H); 3.44 (m, 4H); 5.12 (s, 2H); 6.42 (br, 1H); 7.33 (m, 5H).

Compound 2e—Compound **2e** was synthesized according to the method of **2b**. ^1H NMR (CDCl_3), δ 1.45 (s, 9H); 1.47 (s, 18H); 2.86 (m, 1H); 3.36 (m, 5H); 3.72 (m, 1H); 4.9 (br, 2H).

Compound 11—To a solution of N-CBZ-amido-dPEG4-COOH (1g, 2.5 mmol) and **2b** (1.08 g, 2.5 mmol) in dry chloroform, were added dimethylaminopyridine (cat.) and dicyclohexylcarbodiimide (0.62 g, 3 mmol). The mixture was stirred at room temperature for 48 h. White precipitate was filtered off, and the filtrate was concentrated and applied to HPFC. The crude product was purified on a silica gel flash column with a gradient elution (1%–5% methanol in chloroform). Yield: 1.7 g. TLC: R_f = 0.77 in CHCl_3 -MeOH (9:1). ^1H NMR (CDCl_3), δ 1.45 (s, 9H); 1.47 (s, 18H); 1.51 (m, 3H), 1.64 (m, 3H); 2.45 (t, J = 4.5, 2H); 3.22 (m, 2H); 3.28 (m, 1H); 3.40 (m, 2H), 3.44 (s, 2H), 3.46 (s, 2H); 3.60 (m, 14H), 3.71 (t, J = 4.2, 2H); 5.11 (s, 2H); 5.41 (br, 1H); 6.47 (br, 1H); 7.35 (m, 5H).

Compound 2a—To a solution of **11** (1.58 g, 1.95 mmol) in methanol (35 mL) under nitrogen, were added 10% Pd/C (0.2 g) and ammonium formate (1.4 g, 22 mmol) solution in 1 mL water and 9 mL methanol sequentially. The reaction mixture was vigorously stirred for 4 hours under nitrogen at 40 °C. Pd/C was filtered off over Celite® 545 and the filtrate was evaporated under reduced pressure. The residual was azeotropically dried with toluene. The crude product was purified by HPFC (10% ~ 18% methanol in chloroform). Yield: 1.2 g, 90% wrt **11**. TLC: R_f = 0.1 in chloroform-methanol (9:1). ^1H NMR confirmed the removal of CBZ group from **11**.

Compound 6a—Compound **6a** was synthesized according to the method of **6b**. ^1H NMR (CDCl_3), δ 1.32–1.78 (m, 34H); 2.38 (m, 6H); 2.52 (m, 4H); 2.62 (m, 2H); 2.80 (m, 2H); 3.14–3.30 (m, 18H); 3.37–3.63 (m, 45H); 3.76 (br, 26H); 4.22 (m, 1H); 4.41 (m, 1H).

Compounds 6c–6e—Compounds **6c–6e** were synthesized according to the method of **6b**. Their ^1H -NMR spectra were similar to that of **6b**. The mass of all biotin-tris-NTA (**6a–6e**) were confirmed by MALDIMS using 2,5-dihydroxybenzoic acid as matrix.

Synthesis of His6-peptide

His6pPeptide (N-C sequence: GWGGHHHHHHG) was synthesized on an ABI 433A automated peptide synthesizer (Applied Biosystems) by solid-phase 9H-fluoren-9-ylmethoxycarbonyl chemistry according to standard Fastmoc procedures (15). The peptide was cleaved from the resin with 94% trifluoroacetic acid in the presence of water, ethanedithiol, and triisopropylsilane. The free peptide was separated from the resin by filtration followed by precipitation in cold diethyl ether. Peptide synthesis reagents were obtained from Novabiochem. The molecular weight of His6-Peptides was confirmed by MALDI mass spectrometry (Voyager DE, Applied Biosystems) using alpha-cyano-4-hydroxycinnamic acid as matrix (Calc. 1254.3, found: 1255.4). Lyophilized peptide was stored at –20 °C until use.

Peptide stock solutions of 1 to 5 mM were prepared by dissolution of lyophilized powders in ultrapure water (Milli-Q, Millipore) and filtration through 0.2 μ m PTFE membranes (Whatman). Stock concentrations were determined by tryptophan absorbance at 280 nm as described by Pace (16). Each concentration measurement represented the mean of three dilutions each providing absorbance values between 0.1 and 1.1. Prior to SPR experiment, His6-peptide was further diluted to working concentrations in SPR running buffer.

Surface Plasmon Resonance Experiment

Preparation of tris-NTA sensor chip (Figure 2A): A Series S Sensor Chip SA (Biacore, Piscataway, NJ) was pretreated with two injections of 50 mM NaOH in 1 M NaCl (20 μ L/min, 60 s), then washed with pH 7.4 HBS-P running buffer until the baseline was stabilized. Flow cell #1 was reserved as blank, the remaining flow cells (#2–4) were coated with Biotin-tris-NTA having specific spacers by injection of 20 μ L 100 nM Biotin-tris-NTA (10 μ L/min for 2 min) to capture a 50 RU response. These low density tris-NTA chips were used for the binding kinetics experiments. High density tris-NTA chip (ca. 300 RU) was prepared by saturating the streptavidin chip surface with Biotin-tris-NTA, and was used for the immobilization of His6 protein on the surface.

Binding kinetics (Figure 2B): Various concentrations of the His-tagged molecules were injected to flow over the tris-NTA surfaces, according to the programmed cycles of the following sequence: 1) activation of tris-NTA with 5 mM NiCl_2 , 2) protein injection, 3) Ni(II) removal with 0.3 M EDTA, 4) rinse with 0.25% SDS and 0.5 M NaCl for baseline regeneration and stabilization. Both sample and control measurements were run in duplicates. The raw data were processed with Biaevaluation T100 software, and the kinetic parameters were obtained by global fitting the experimental curves with 1:1 Langmuir binding model. The quality of the fitting was estimated by T value and the residue (χ^2). $T(k) = k/\text{SE}(k)$, where k is a fitted parameter such as k_a , $\text{SE}(k)$ is the standard error of k . A good fit should have a small residue and a large T value (17).

RESULTS

Synthesis of Biotin-Tris-NTA

Biotin-tris-NTAs were synthesized according to previously published method (11) (Scheme 1). Briefly, the amino group of NTA *tert*-butyl ester (**2**) having various spacers was coupled to carboxyl acids of benzyloxycarbonyl protected lysine NTA scaffold (**1**). Biotin was conjugated to tris-NTA after the removal of the benzyloxycarbonyl group so that the final product (**6**) can be immobilized on a streptavidin sensor chip for the surface plasmon resonance (SPR) measurement. In addition to the original lysine spacer (**6b**), four variants were introduced by either reducing the number of methylene groups (**6c–e**) or extending the lysine spacer with a flexible polyethylene oxide chain (**6a**). These alterations have the effect of varying the distance between adjacent mono-NTAs in the cluster.

Binding Parameters

The binding kinetics of tris-NTA/His6 complex were measured on a Biacore T100 SPR instrument. A customized tris-NTA chip was prepared by coating a Biacore streptavidin chip with Biotin-tris-NTA at low surface density (around 50 response units, about 1/6 of the chip surface capacity, illustrated in Figure 2a). The surface density of tris-NTA was kept low to avoid the avidity effect but high enough to allow a good measurement (18). The mass transport effect on measured affinity was suppressed by using relatively high flow rate (50 μ L/min) and was automatically isolated by the Biaevaluation T100 program. Typical SPR sensorgrams of the tris-NTA/His6 complex are shown in Figure 3. All the data fit a simple

1:1 binding model well with negligible residue and satisfactory T values (> 100). The binding parameters of tris-NTA with His6-tagged molecules are summarized in Table 1.

The kinetics data (Table 1) showed that the length of spacer in tris-NTA has a significant effect on the binding affinity. The general trend is higher affinity and lower instant off rate for shorter spacer. The apparent binding affinity toward the same tris-NTA varies for different His6-tagged proteins and His6-peptide which may relate to the accessibility of the His6-tag. Sub-nanomolar affinity was reached by **6d**, and no further improvement was achieved by **6e**.

DISCUSSION

We synthesized a series of biotin-tris-NTA to study the effect of spacer on the binding affinity of tris-NTA nickel (II) complex toward His6 tagged molecules. The introduction of biotin moiety in the tris-NTA conjugate allows the preparation of a customized low surface density tris-NTA chip for the SPR measurement of the binding kinetics. SPR is a powerful and valid technique to measure molecular interactions in real time (19). We have used SPR in a previous study to measure the interaction between tris-NTA and His6 tagged proteins; the binding affinity was consistent with the data determined by the isothermal titration calorimetry (9,11).

To quantify the interaction between multivalent binding pairs, it is important to keep the surface density low to avoid the avidity effect since the apparent affinity of His6 with mono-NTA was dependent on the surface density (20). The advantage of a tris-NTA surface over a mono-NTA surface is that tris-NTA can provide a local high “effective concentration” even at low surface density. In our experiment, the surface density is about one sixth of the total capacity of the chip and the experimental kinetics data can be globally fitted by a simple 1:1 binding model with very low residue and satisfactory T values (> 100, indicating less than 1% uncertainty of the calculated data). In addition to low surface density, low analyte concentration is also required to minimize the undesired multi-site interactions such as two His6 tags binding to one tris-NTA. Thus, a concentration that leads to maximum signal is not suitable for the 1:1 binding kinetics study on a multivalent binding pair as tris-NTA and His6. After systematic evaluation of the SPR conditions, we determined that 1~10 nM was a good concentration range for the kinetics study. Although quantification of multivalent interactions is still a challenge, we acquired the kinetic data as accurate as possible through the combination of a sensitive instrument (Biacore T100) and careful experiment design; this included low surface density, low analyte concentration and a high flow rate.

The focus of current research is to investigate the influence of the spacer length on the binding affinity. We have synthesized tris-NTAs having five different spacers and included five His6-tagged molecules in order to determine if there is a general trend. Although the K_d value varies for different His6 tagged molecules, the trend is clear; as the spacer gets shorter, the affinity is higher. Different proteins have different binding affinities; these differences may arise from the extent of exposure of the His6-tag and other non-specific interactions that are idiosyncratic for each protein. For example, TSG6 had an abnormally high affinity (0.02 nM, exceeded the detection limit of Biacore T100) toward **6c**. This cannot simply be attributed to the His6/tris-NTA interaction but is probably caused by other structural features of the protein in the vicinity of the local structure of the His6.

The His6-peptide may provide a closer model of the binding between His6 and tris-NTA. The high affinity of **6d** may be partly attributed to the short spacer that results in less conformational entropic cost of association than the flexible long spacers. When the spacer gets even shorter, such as in **6e**, the enthalpy of binding may become less favorable due to

the increased steric hindrance and may offset the entropic effect. Thermodynamic studies of the tris-NTA/His6 binding by SPR at various temperatures (data not shown) were inconclusive and we were unable to determine whether the entropy or enthalpy of binding was the predominant factor leading to the increased affinity. These measurements are most certainly complicated by the conformational change of the His6-tagged protein at different temperatures. Thus, it is difficult to quantify entropy and enthalpy changes of binding in such a complex system by the SPR technique. Nevertheless, it is clear the binding affinity of tris-NTA to His6-tag can be substantially improved by employing a shorter spacer.

Formation of a stable complex is essential to the broad applications of tris-NTA/His6 technology. One major problem in protein-protein interaction studies using the commercial SPR NTA chip is fast baseline drift due to the dissociation of protein immobilized on the surface through mono-NTA/His6 interaction. The new high-density tris-NTA chip (such as coated with **6e**) enables reversible yet stable immobilization of His6-tagged protein with a stable baseline. The high density tris-NTA is now broadly used by researchers at University of California San Francisco for SPR analysis of various proteins (21). Derivatives of high affinity tris-NTA can also be used to attach His6-tagged molecules to nanoparticles such as carbon nanotubes, gold particles, liposomes, magnetic particles, micelles and quantum dots.

In conclusion, we have demonstrated that sub-nanomolar affinity to a His6 tag can be achieved by tris-NTA where the individual NTAs are separated by short spacers. The development of these high affinity binding pairs opens the avenue to broader applications of His6-tagged molecules with enhanced stability.

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Figure 1. Schematic illustration of tris-NTA/His6 tag binding pair. (A) Functional unit, (B) Scaffold, (C) Spacer, (D) Structure of NTA/Ni²⁺/His2. The flexibility and the length of the spacer play an important role in the overall binding affinity of tris-NTA toward His6 tag.

Figure 2. Measurement of binding kinetics by surface plasmon resonance using a tris-NTA chip. (A): Tris-NTA chip was prepared by coating a streptavidin chip with biotin-tris-NTA at low surface density. (B): Representative kinetics experiment cycle.

Figure 3.

Double-referenced SPR sensorgrams of His6-mKate binding to tris-NTA having various spacer at a series of concentrations (1~10 nM at 1 nM increment excluding 7 nM). Experimental data were globally fitted with a 1:1 binding model. Black line: experimental data; red line: fitted data. The precision of the fitting was estimated by T value that can be interpreted as signal to noise ratio. A T value of 100 corresponds to 1% uncertainty. (A): mKate/**6a**, T (ka): 100, T (kd): 110. (B): mKate/**6c**, T (ka): 180, T (kd): 240. (C): mKate/**6d**, T (ka): 150, T (kd): 150.

Scheme 1.
General synthetic route for biotin-tris-NTA

Scheme 2.

Synthesis of amino-NTA(O-tBu). (a): Benzyl chloroformate (1 equiv.), pH 7.0 phosphate buffer, r.t.; (b): 2-methylpropene, conc. H₂SO₄, dioxane, r.t.; (c): tert-butyl bromoacetate (4 equiv.), DIPEA, DMF, 55 °C; (d): HCO₂NH₄ (6 equiv.), 10% Pd/C, 90% methanol, 40 °C.

Scheme 3.

Synthesis of **2a**. (a): DCC (1.2 equiv.), DMAP, dry CHCl_3 , r.t.; (b): HCO_2NH_4 (6 equiv.), 10% Pd/C, 90% methanol, 40°C .

Table 1

Binding Parameters of Tris-NTA/His6 Complexes^a

Tris-NTA	AR-NTD ^c			yCD ^d			TSG6 ^e			His6-Peptide ^f			mKate ^g		
(spacer) ^h	k _a ^h	k _d ⁱ	K _D ^j	k _a	k _d	K _D	k _a	k _d	K _D	k _a	k _d	K _D	k _a	k _d	K _D
6a (>4) ^k	0.94	1.29	13.7	3.11	12.3	39.4	6.89	4.84	7.03	3.09	3.97	12.8	5.38	1.53	2.84
6b (4)	0.81	1.40	17.3	1.19	0.83	6.97	19.4	5.78	3.0	1.86	1.85	9.95	ND	ND	ND
6c (3)	1.44	0.27	1.87	1.97	0.59	3.02	3.92	0.008	0.02	2.19	0.72	3.29	10.6	0.43	0.40
6d (2)	ND	ND	ND	ND	ND	ND	ND	ND	ND	35.8	0.64	0.18	10.3	0.20	0.21
6e (1)	ND	ND	ND	ND	ND	ND	ND	ND	ND	33.8	0.62	0.18	ND	ND	ND

^aMeasurements were carried out at 25 °C on a Biacore T100 SPR machine, and were repeated at least once. Data were analyzed by Biaevaluation T100 software with a global fit of 1:1 binding model. All fitted curves had low residue and satisfactory T value (> 100). All protein ligands tested have a C-terminal His6-tag.

^bNumber of methylene groups in the spacer.

^cAndrogen receptor N-terminal domain, MW: 21 K dalton.

^dYeast cytosine deaminase (22), MW: 19.7 K dalton.

^eTumor necrosis factor alpha-stimulated gene-6 (22), MW: 13.5 K dalton.

^fHis6-tagged peptide: GWGGHHHHHGG.

^gMonomeric Kaushka, a far-red fluorescent protein (23), MW: 27 K dalton.

^hk_a Instant association rate constant, 10⁵ M⁻¹ s⁻¹.

ⁱk_d Instant dissociation rate constant, 10⁻³ s⁻¹.

^jK_D Equilibrium dissociation constant, 10⁻⁹ M.

^kSpacer:(CH₂CH₂O)₄CH₂CH₂C(O)NH(CH₂)₄.