Accelerating phage-display library selection by reversible and site-specific biotinylation

Akiko Koide[1], John Wojcik[1], Ryan N. Gilbreth[1], Annett Reichel[2], Jacob Piehler[2†] and Shohei Koide[1,3]

[1]Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637, USA and [2]Division of Biophysics, University of Osnabrück, Osnabrück, Germany

[3]To whom correspondence should be addressed.
E-mail: skoide@uchicago.edu

Immobilization of a target molecule to a solid support is an indispensable step in phage display library sorting. Here we describe an immobilization method that addresses shortcomings of existing strategies. Our method is based on the use of a polyhistidine-tagged (His-tagged) target molecule and [BT]tris-NTA, a high-affinity capture reagent for His-tags that also contains a biotin moiety. [BT]tris-NTA provides a stable and reversible linkage between a His-tag and a streptavidin-coated solid support. Because His-tags are the *de facto* standard for recombinant protein purification, this method dramatically simplifies target preparation for phage display library sorting. Here, we demonstrate the utility of this method by selecting high-affinity binding proteins based on the fibronectin type III (FN3) scaffold to two His-tagged protein targets, yeast small ubiquitin-like modifier and maltose-binding protein. Notably, a significant number of FN3 clones binding either targets selected using the new immobilization method exhibited only very weak binding when the same target was immobilized by coating on a polystyrene surface. This suggests that the His-tag-mediated immobilization exposes epitopes that are masked by commonly used passive adsorption methods. Together, these results establish a method with the potential to streamline and enhance many binding-protein engineering experiments.

Keywords: directed evolution/high-throughput selection/monobodies/synthetic binding proteins

Phage display is a widely used method for the selection of protein variants from a large library based, most commonly, on their ability to bind to a target molecule of interest (Smith, 1985; Sidhu and Koide, 2007). In order to separate phages that bind to the target from those that do not, the target molecule needs to be immobilized on a solid support (Sidhu *et al.*, 2000). When one wishes to select 'binders' to the native form of a target protein, it is important to immobilize the target in such a way that it is not denatured and most of its surfaces remain accessible. To maximize the recovery of phages that bind to the immobilized target, but not to other materials present in the apparatus, it is advantageous

[†]Inquiry regarding [BT]tris-NTA availability should be addressed to J.P.
E-mail: piehler@uos.de.

for the linkage between the target and the solid support to be conditionally reversible, so that phages specifically bound to the target can be selectively released from the solid support (Fellouse *et al.*, 2007).

A common immobilization method is passive, non-specific adsorption to a modified polystyrene-based surface, usually in the format of the wells of a microtiter plate. Despite its widespread use and proven success in generating binding proteins, it is not clear what form the immobilized protein assumes on the polystyrene surface, and it is possible that some proteins are denatured upon immobilization and/or immobilized in a certain preferred orientation, which render a subset of epitopes inaccessible (Butler *et al.*, 1992). Moreover, phages bound to the immobilized target on polystyrene surfaces are usually recovered by disrupting the molecular interactions with harsh solutions such as acid or denaturant (Sidhu *et al.*, 2000). This type of elution is non-specific, and recovered phages include those that bind to the solid support. Thus, non-specific elution may lead to the enrichment of 'background' binders that do not specifically bind to the target, thereby reducing the overall efficiency of identifying true target-binding clones.

One way to address the limitations imposed by passive adsorption is to attach a biotin moiety to the target protein in a manner that does not affect the structural integrity of the target. Such modified targets can then be immobilized on a surface coated with a biotin-binding protein such as streptavidin or avidin. This method exploits the exceptionally high affinity and rapid kinetics of the biotin–(strept)avidin interaction (Stayton *et al.*, 1999). Once formed, the interaction is essentially irreversible. This tight interaction allows for phage display selection at a very low target concentration, which is useful for the selection of high-affinity binders.

Biotin is most commonly attached to a protein either through chemical modification, commonly at amine or sulfhydryl groups, or through the construction of a fusion protein with an enzymatic biotinylation tag. Chemical biotinylation reagents include those that can later be cleaved with a reducing reagent, enabling specific release of an immobilized target (Griffiths *et al.*, 1994; Fellouse *et al.*, 2007). Utilizing such a cleavable biotinylation reagent, we have previously established a high-throughput pipeline for the selection of phage-displayed antibodies (Fellouse *et al.*, 2007; Koide *et al.*, 2007). However, in chemical biotinylation, one has to optimize reaction conditions, so that the target protein is biotinylated at a small number of sites, or ideally at a single site. Biotinylation of a protein at many sites may drastically change the target's surface properties, alter its conformation or result in the selection of binding proteins that recognize only an over-biotinylated form of the target. Further, biotinylation often reduces the solubility of the target protein due to the hydrophobic nature of the biotin moiety. Also, the cleavable biotinylation reagents that are currently commercially available utilize reductive cleavage of a disulfide bond. This necessitates storage and handling of a protein biotinylated in

this manner under oxidizing conditions. Accordingly, these reagents are incompatible with a target protein that contains free cysteine residues that need to be kept reduced, and such reactive cysteines that are non-essential must be eliminated via chemical modification or mutagenesis prior to biotinylation. Therefore, although we found our procedures utilizing a cleavable biotinylation reagent to be highly effective in producing high-affinity binders, they require multiple steps and careful optimization of target modification as summarized in Fig. 1A.

Enzymatic biotinylation provides an alternative to these chemical biotinylation methods. Biotin ligase recognizes a specific sequence ('biotin acceptor domain') and attaches biotin to a Lys residue in the sequence (Schatz, 1993). This process can be exploited either *in vivo* or *in vitro*, but producing a new fusion protein adds an extra step in target protein production. Also the biotin moiety attached by biotin ligase is not cleavable, making it difficult to specifically release the immobilized target.

It is clear from these considerations that although biotinylation is a highly effective immobilization method that can retain the structural integrity of a target protein, it has significant limitations. Therefore, a new, reversible immobilization method that does not involve covalent modification but retains a high-affinity linkage would significantly improve the efficiency of phage-display library selection and potentially expand the range of target proteins that can be used in such projects.
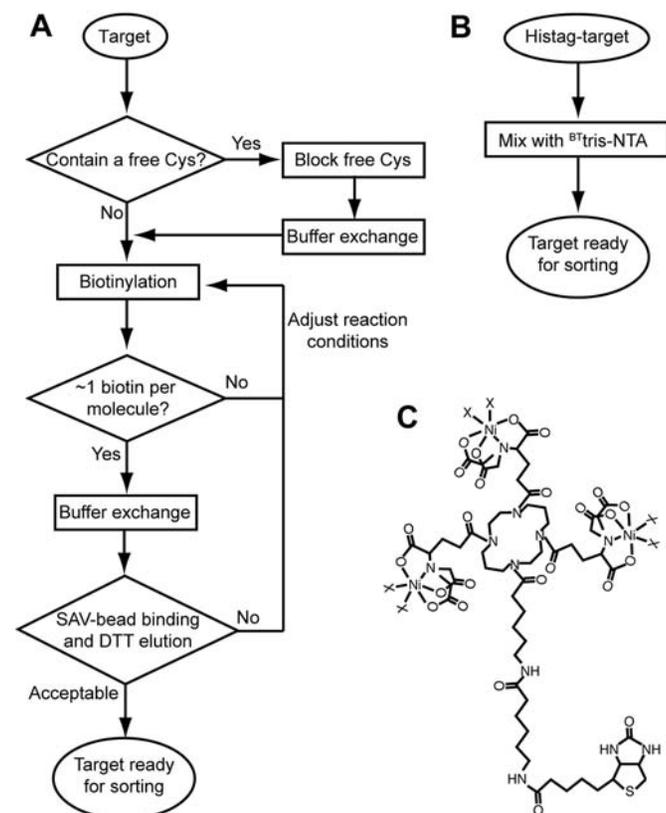
Polyhistidine tags (His-tags) have become the standard fusion tag for protein purification by immobilized metal ion affinity chromatography. For example, virtually all proteins in large-scale structural genomics centers are produced, at least initially, as a His-tag fusion (Graslund *et al.*, 2008). The tag's small size and efficacy in a wide range of solution conditions are ideally suited for many applications. Thus, it would be highly beneficial if a method were available for directed high-affinity immobilization of His-tag proteins. The standard His-tag ligands, such as the nitrilotriacetic acid (NTA) moiety loaded with Ni(II) ions, bind with low affinity ($\sim$10 µM) (Dorn *et al.*, 1998). Stable immobilization of His-tagged proteins on NTA surfaces requires a high density of the NTA molecule (Lata *et al.*, 2005) to allow simultaneous interactions of the His-tag with several chelated metal ions. These high densities of immobilized transition metal ions, however, lead to increased non-specific interactions with the excess immobilized Ni(II) ions (Lata *et al.*, 2005). Although there are anti-His-tag antibodies, which circumvent this problem, their high costs and relatively low affinity limit their effectiveness.

Recently, Lata *et al.* (2005) developed a series of His-tag ligands that have multiple NTA moieties. These ligands can form stoichiometrically well-defined multivalent interactions with a His-tag, and thus offer high affinity binding in solution with a K_d in the low nanomolar range. Among these, tris-NTA contains three NTA moieties that can simultaneously bind to a maximum of six histidine side chains with a K_d of $\sim$2 nM for a 6-histidine tag and 0.14 nM for a 10-histidine tag (Lata *et al.*, 2005). Further, Reichel *et al.* (2007) developed a biotinylated form of tris-NTA called [BT]tris-NTA with the initial motivation of labeling a His-tag protein with biotin for downstream detection. The high-affinity linkage between a His-tagged protein and [BT]tris-NTA in solution allows for the capture of the complex with a solid support presenting a biotin-binding protein, such as streptavidin-coated magnetic beads. By this strategy, a His-tagged target can be specifically captured, while avoiding presentation of excess of immobilized Ni(II) ions susceptible to non-specific interaction as in other His-tag capture methods. Moreover, the association of the biotin–streptavidin complex is much faster than that of the His-tag with Ni-NTA, making capture of the [BT]tris-NTA labeled target more rapid and efficient than immobilization directly through the His-tag.

The ability of [BT]tris-NTA to rapidly capture a His-tag protein on solid support with high affinity and selectivity is an attractive feature for its potential use in phage display selection (Fig. 1B). Furthermore, the linkage between tris-NTA and a His-tag can be disrupted under physiological conditions with imidazole or EDTA, providing a convenient means for mild and selective release of the captured protein. Because low target concentration is necessary for the selection of high-affinity binders and selection processes involve vigorous washing, it was not clear whether the non-covalent [BT]tris-NTA label could be used in such selection experiments. Moreover, selection with [BT]tris-NTA might enrich sequences that are rich in histidine that bind to the tris-NTA moiety but not to the actual target. Therefore, in this work, we tested whether [BT]tris-NTA could be used for the selection of binding proteins from a phage display library.

Fig. 1. (**A**) A flowchart outlining steps required for target preparation using a chemical biotinylation reagent (Fellouse *et al.*, 2007; Koide *et al.*, 2007). Note that the method presented in this work (**B**) completely eliminates these steps. (**C**) The chemical structure of [BT]tris-NTA (Reichel *et al.*, 2007). The X's denote Ni ligands such as His and imidazole.
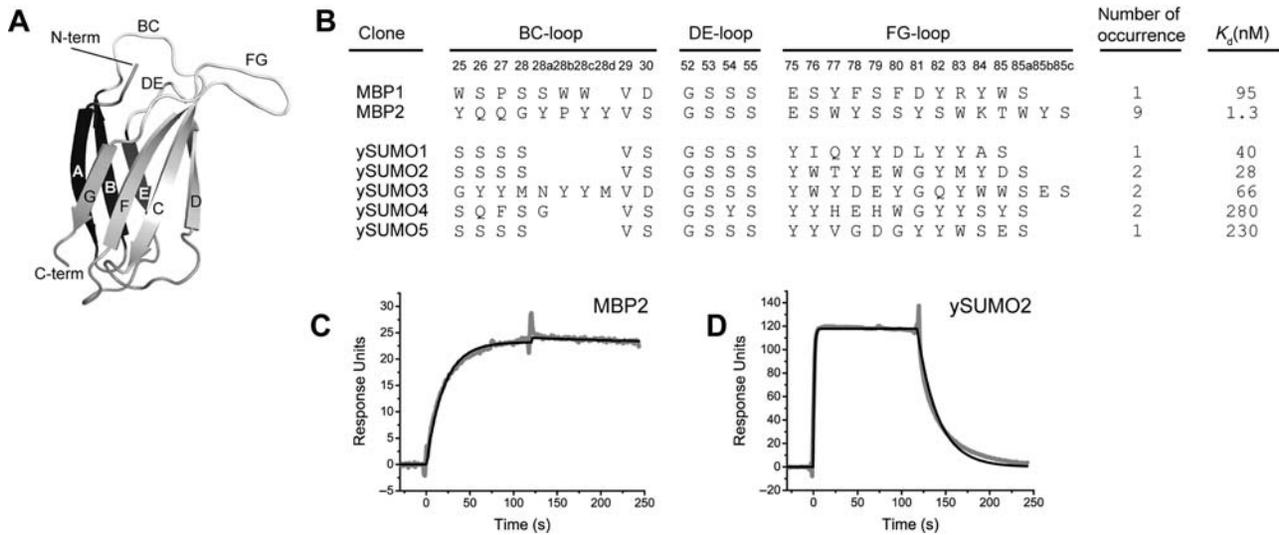
Fig. 2. (**A**) A schematic drawing of the monobody scaffold. β-Strands A–G and the three loops that are diversified in the library are indicated. (**B**) The amino acid sequences and affinity of monobodies obtained from library selection. The sequences for the three loops are shown, with the numbering of Main *et al.* (1992). K_d values determined using SPR are shown. (**C** and **D**) SPR sensorgrams for the interactions of MBP and monobody M4 (C) and ySUMO and monobody Y12. The His-tagged monodies were immobilized on an NTA-chip and the association and dissociation of MBP and ySUMO were monitored.

Materials and methods

[BT]Tris-NTA synthesis

[BT]Tris-NTA was synthesized as described previously (Lata *et al.*, 2005; Reichel *et al.*, 2007).

Protein preparation

MBP and yeast small ubiquitin-like modifier (ySUMO) were expressed as His$_{10}$- and His$_6$-tagged proteins, respectively, using pHFT1 and pHFT2 vectors (Huang *et al.*, 2006; Koide *et al.*, 2007). Selected monobodies (FN3-based binding proteins) were also expressed as His$_{10}$-tagged proteins. These proteins were purified using Ni-affinity chromatography (Ni-chelating Sepharose, Amersham). For surface plasmon resonance (SPR) measurements, the His-tag of the target proteins was cleaved using TEV protease and removed using Ni-affinity chromatography.

Phage display library sorting

General procedures for phage preparation and analysis of individual clones with phage ELISA have been described previously (Sidhu *et al.*, 2000; Koide and Koide, 2007). A phage display library of monobodies was similar to that described previously (Gilbreth *et al.*, 2008), but two surface loops (the BC and FG loops) were diversified using the following amino acid composition: Tyr, 30%; Ser, 15%; Gly, 10%; Phe, 5%; Trp, 5% and all other except for Cys, 2.5% each.

Library sorting was performed at room temperature essentially following published procedures (Fellouse *et al.*, 2007; Koide *et al.*, 2007; Gilbreth *et al.*, 2008) except that His-tagged targets complexed with [BT]tris-NTA were used. His-tagged proteins were mixed with [BT]tris-NTA in TBS (50 mM Tris–HCl buffer, pH 7.5, 150 mM NaCl) and incubate at room temperature for 30 min before use.

In the first round, a target-[BT]tris-NTA complex was mixed with a sufficient amount of streptavidin-conjugated magnetic beads (Streptavidin MagneSphere Pramagnetic Particles;

Promega) in TBS. To this target solution, $10^{12–13}$ phagemids suspended in 1 ml TBS plus 0.5% BSA were added, and the solution was mixed and incubated for 15 min at room temperature. After washing the beads twice with TBS containing 0.05% Tween20 (TBST), the beads suspension containing bound phagemids was added to fresh *Escherichia coli* XL1-Blue culture. Phagemids were amplified in the presence of 0.1 mM IPTG. In the second and later rounds, library sorting was performed using a Kingfisher instrument (Thermo Scientific) (Fellouse *et al.*, 2007; Koide *et al.*, 2007). Phagemids amplified from the previous round were incubated with a target-[BT]tris-NTA complex in TBS containing 0.5% BSA, and then captured by streptavidin-conjugated magnetic beads. After a total of five washing steps with 100 μl TBST with BSA (20 s per step), phagemids bound to the target protein were eluted by incubating the beads with 400 mM imidazole in TBS or 10 mM EDTA in TBS for 10 min. Recovered phages were then amplified.

SPR measurements

A monobody containing a His$_{10}$ tag was immobilized on a NTA-chip (BIAcore) in a BIAcore 2000 instrument, and binding of targets (10–100 nM) was monitored. Sensorgrams were analyzed with the BIAEvaluation program (BIAcore).

Results and discussion

[BT]Tris-NTA effectively immobilizes His-tagged proteins

We first performed a set of mock selection experiments to examine if the interaction of [BT]tris-NTA and a histidine-tagged protein is strong enough to survive the vigorous washing steps used in our procedures. We used His$_{10}$-tagged maltose-binding protein (MBP) as a target and phages displaying an MBP-binding protein, monobody MBP74 (Koide *et al.*, 2007). Monobodies are synthetic binding proteins based on the fibronectin type III (FN3) scaffold (Koide *et al.*, 1998) (Fig. 2A). Four types of mock selection were performed, with and without [BT]tris-NTA and with and
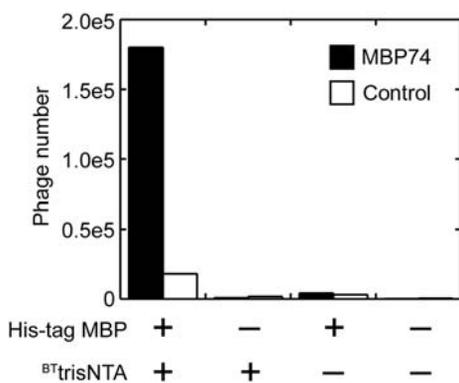
Fig. 3. Recovery of phages displaying an MBP-binding monobody (filled bar) from selection with His-tagged MBP immobilized to magnetic beads through ^{BT}tris-NTA (the leftmost column). As a negative control, phages displaying a non-functional, 'shaved' FNfn10 in which residues in the BC and FG loops have been replaced with Ser (Koide *et al.*, 2007) were used (open bar). The numbers of recovered phages are shown. The three right columns represent control experiments in which ^{BT}tris-NTA and/or the target were omitted.

without His-tag-MBP. The MBP-binding phages and control phages were, respectively, incubated with these targets, captured with streptavidin-coated magnetic beads, washed vigorously and released with imidazole. Phages displaying the MBP-binding monobody were recovered efficiently only when both ^{BT}tris-NTA and His-tag MBP were included (Fig. 3). These results demonstrate that the interaction between ^{BT}tris-NTA and the His-tag-MBP was maintained throughout the selection process and thus the combination of ^{BT}tris-NTA and a His-tagged protein may be used as an alternative to a covalently biotinylated protein.

^{BT}*Tris-NTA can be used in phage-display library sorting*

We then proceeded to selection of monobodies from a naïve library. His$_{10}$-tagged MBP and His$_6$-tagged ySUMO were used as targets. We have previously selected monobodies to these targets using chemically biotinylated proteins (Koide *et al.*, 2007). We have adapted the selection method established previously (Koide *et al.*, 2007; Gilbreth *et al.*, 2008) with small modifications. In place of chemically biotinylated proteins, the His-tagged proteins were mixed with the stoichiometric amount of ^{BT}tris-NTA, and instead of recovering the target-phage complex by cleaving the biotinylation reagent with dithiothreitol (DTT), we eluted the target-phage complex with 400 mM imidazole. The target concentration was 200 nM for the first round and 100 nM for the second through fourth round. In the fifth round, we performed selection using 100, 20 nM and no target, and determined the number of recovered phages. The ratio of the phage number recovered in the presence of target over that in the absence of target, termed 'enrichment ratio', is a good indicator of whether the selection succeeded or not. If the enrichment is 10 or higher, usually a majority of the clones from the enriched pool bind to the target (Fellouse *et al.*, 2007; Koide *et al.*, 2007). For the selection with MBP, we obtained an enrichment ratio of 12 and 11 with 100 and 20 nM MBP, respectively. For the selection with ySUMO, the enrichment was 7.7 and 1.4 with 100 and 20 nM ySUMO, respectively.

Next, we randomly selected 12 clones each from the enriched pool sorted with 20 nM MBP and that with 100 nM

ySUMO. Ten of 12 clones from the MBP-binding pool and 9 of 12 clones of the ySUMO-binding pool bound to their respective target as tested using phage ELISA. This was consistent with the good enrichment ratios observed for these sorting experiments. Sequence determination revealed one dominant clone and a second, additional clone for MBP and five clones for ySUMO (Fig. 2B). Importantly, the recovered phage clones bound to their cognate targets but not to ^{BT}tris-NTA and no clones with more than two His residues in the diversified loops were found, although the library design can encode oligo His sequences.

These MBP- and ySUMO-binding monobodies were then prepared as free proteins and their binding affinity was measured using SPR. The dissociation constants for these monobodies are in the low and mid nanomolar ranges (Fig. 2), clearly demonstrating the ability to select high-affinity binding proteins using ^{BT}tris-NTA and a His-tag protein.

His$_{10}$-tag is more effective than His$_6$-tag

In the above experiments, selection using 20 nM ySUMO did not yield significant enrichment of binding clones. In contrast, MBP binders were selected with 20 nM target. The failure to enrich ySUMO binders was not due to a lack of high-affinity clones in the naïve library, because we selected ySUMO binders with a K_d as low as 28 nM from sorting with 100 nM ySUMO (Fig. 2B). Such strong binders should have been selected with 20 nM target. A major difference between the MBP and ySUMO targets is that MBP had a His$_{10}$-tag whereas ySUMO had a His$_6$-tag. We suspected that the linkage of ^{BT}tris-NTA and the His$_6$-tag was not stably maintained at the concentration of 20 nM, leading to the release of ^{BT}tris-NTA from the target-binder complexes during sorting. Although the affinity of ^{BT}tris-NTA and His$_6$ in K_d was determined to be 2 nM (Lata *et al.*, 2005), the complex may dissociate during vigorous washing steps in selection.

In order to investigate if the His$_6$- and His$_{10}$-tags give different results in library sorting, we performed selection with His$_6$-ySUMO and His$_{10}$-ySUMO. The K_d of ^{BT}tris-NTA and His$_{10}$ is 0.14 nM (Lata *et al.*, 2005), which is ~15 times lower than that of ^{BT}tris-NTA and His$_6$. We also included covalently biotinylated ySUMO as a control whose biotin does not dissociate during binding and washing steps. Selection of ySUMO-binders was performed using target concentration of 200 nM for the first round and 100 nM for the second and third rounds. In the fourth round, we performed selection using 100, 20 nM and no target, and determined the enrichment ratios. In this set of selections, we used 10 mM EDTA in TBS as an elution reagent because this method reduces the recovery of non-specific binders compared with imidazole elution. For chemically biotinylated ySUMO, we used 100 mM DTT in 20 mM Tris–Cl (pH8) that cleaves a disulfide linkage in the biotinylation reagent.

The His$_{10}$- and His$_6$-tags showed a significant difference in the effectiveness in library sorting. The outcome of the three sets of sorting was evaluated by determining the enrichment ratio. The pool sorted with chemically biotinylated-ySUMO gave enrichment ratios of 340 and 230 with 100 and 20 nM target concentrations, respectively. The enrichment ratios were 130 and 100 with 100 and 20 nM

His$_{10}$-ySUMO. In contrast, His$_6$-ySUMO gave enrichment ratios of only 6 and 1. These differences in enrichment ratio indicate that, although both His$_6$- and His$_{10}$-tagged proteins can be used as targets in conjunction with ^{BT}tris-NTA, His$_{10}$ is more efficient in immobilizing a target and accordingly in enriching binders over the course of a multiple-round selection.

The above data made it clear that over the course of a multiple-round selection, the use of a ^{BT}trisNTA–His$_6$-ySUMO complex resulted in reduced enrichment. It remained quantitatively unclear, however, how drastically the use of the ^{BT}trisNTA–His$_6$-ySUMO complex affected the output of a round of selection compared with the other immobilization methods. In an experiment complementary to the above library sorting, we compared the enrichment ratios obtained for each immobilization method when the input phage population and number were identical across all three methods. For this 'mock fourth round', we used the amplified phage after three rounds of selection with the chemically biotinylated ySUMO. Just as in the original selection, chemically biotinylated ySUMO gave enrichment of 340 and 230 with 100 and 20 nM target concentrations. His$_{10}$-ySUMO gave identical enrichment ratios. In contrast, His$_6$-ySUMO gave enrichment ratios of only 35 and 25, respectively. These results further confirmed that the low stability of the ^{BT}trisNTA–His$_6$-ySUMO complex resulted in a significantly lower efficiency in the recovery of target-binding clones, which is likely to have contributed to the very low level of binder enrichment when His$_6$-ySUMO was used throughout multiple rounds of library sorting.

Because many potential targets for phage display selection contain free cysteine residues, we also tested if ^{BT}tris-NTA can be used in library sorting in the presence of the common reducing agent DTT. Because DTT has metal chelating ability, it could potentially act as a competitor for a His-tag protein. One millimolar DTT did not significantly reduce the binding of MBP- and ySUMO-binding phages to their respective targets (Fig. 4), suggesting that our method can also be used to select binders to a His-tagged target containing a reactive Cys residue that needs to be kept reduced in the presence of a reducing reagent such as DTT.

His-tag-mediated immobilization exposes epitopes masked in passive adsorption

To test if the His-tag-mediated immobilization and standard passive adsorption result in a qualitative difference in the state and accessibility of immobilized targets, we first tested binding of the two MBP-binding FN3 monobodies (Fig. 2B) to the antigen immobilized using different methods: (i) His-tagged MBP and ^{BT}trisNTA, (ii) passive adsorption of His-tagged MBP and (iii) chemical biotinylation of His-tag cleaved MBP. Both monobodies showed strong binding to the antigen immobilized using His-tag and ^{BT}trisNTA and to a lower degree to the chemically biotinylated antigen (Fig. 5A). In contrast, neither showed significant binding to the same antigen directly coated to the polystyrene surface. This lack of binding was not due to low levels of immobilization, because similar amounts of the antigen were detected by an anti-FLAG antibody (Fig. 5B). Also, the strong binding of the monobodies to the His-tag cleaved MBP eliminates the possibility that the monobody–MBP interaction is somehow mediated by the His-tag. Together, these results indicate that passive adsorption immobilizes MBP in a manner that renders the epitope of these monobodies inaccessible.

Next, in order to see if the weak binding to passively immobilized antigen is a specific feature for MBP, we tested FN3 monobody clones that had been selected from the above ySUMO-sorting experiments (Fig. 5) using both His-tag-mediated and direct immobilization methods. Similar to the

Fig. 4. Effect of DTT on the immobilization of His-tagged targets tested via binding of monobody-displaying phages. His-tagged MBP (for the M4 monobody) and ySUMO (for the Y12 monobody) were immobilized to neutravidin-coated wells of a microtiter plate and binding of MBP- and ySUMO-binding monobodies displayed on phages were tested in the presence and absence of 1 mM DTT. Bound phages were detected with an anti-phage antibody. The identities of the two monobody clones are shown in Fig. 2.

Fig. 5. Comparison of binding of FN3 monobody clones to MBP and ySUMO immobilized via His-tag and ^{BT}trisNTA and via direct coating. (**A**) Phage ELISA readout for binding of the MBP-1 and -2 monobodies (Fig. 2B) to His$_{10}$-MBP directly coated to polystyrene surface (gray bars), to His$_{10}$-MBP immobilized via ^{BT}trisNTA (white bars) and to MBP immobilized via chemical biotinylation (striped bars). The black bars show binding data to the surface containing ^{BT}trisNTA but not His$_{10}$-MBP. (**B**) ELISA readout with anti-FLAG antibody showing the amounts of immobilized MBP. Symbols are the same as in (A). (**C**) Phage ELISA readout for 12 independent clones of ySUMO-binding FN3 monobodies binding to His$_{10}$-ySUMO immobilized via direct coating (gray bars) and via ^{BT}trisNTA (white bars). The black bars are the negative control as in (A).

results with MBP, while all clones bound to His_{10}-ySUMO immobilized through ^{BT}trisNTA equally well, a subset exhibited very weak binding to His_{10}-ySUMO directly coated on polystyrene surface (e.g. clone 3 in Fig. 5C). These results suggest that ySUMO is immobilized on polystyrene surface in a non-random fashion, which sequesters a subset of epitopes or that immobilization via adsorption somehow alters the ySUMO structure. Because ^{BT}trisNTA immobilizes the His-tag portion but not ySUMO itself, the entire ySUMO surfaces should be accessible by binding proteins, which allows for the selection of broader types of binders.

In conclusion, our results have demonstrated that the combination of ^{BT}tris-NTA and a His-tagged target can significantly simplify the target preparation process for phage-display library selection. The method also presents an antigen in a manner that minimizes sequestration of epitopes. We have established a generic protocol for rapid and reversible biotinylation of target proteins, which does not require optimization for each target and enables mild and selective phage elution. Because the new method can be used in the presence of DTT, it also expands the range of targets to which phage display selection with reversible target immobilization can be applied. In our experience in performing high-throughput sorting of phage display libraries using chemical biotinylation, target preparation (Fig. 1A) is a major bottleneck. As most recombinant proteins are made with a His-tag, and are thus already properly formatted, we expect that the new sorting method using ^{BT}tris-NTA presented here will streamline library sorting. These results should be broadly applicable to all molecular display techniques, including phage display, mRNA/ribosome display (Lipovsek and Pluckthun, 2004) and yeast surface display (Boder and Wittrup, 2000).

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