Improved affinity coupling for antibody microarrays: Engineering of double-(His)$_6$-tagged single framework recombinant antibody fragments

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Antibody-based microarray is a novel technology with great promise in biomedicine that will provide unique means to perform global proteome analysis. In the process of designing the high-density antibody microarrays required, several critical key issues have been identified that remain to be resolved. In particular, there is a great need for specific and selective approaches enabling non-purified probes to be directly purified, orientated and coupled in a generic one-step procedure directly on the chip. In this study, we report on the successful design of affinity-tagged human recombinant single-chain fragment variable antibody fragments for improved affinity coupling in array applications. By replacing the standard single-histidine (His)$_6$-tag with a consecutive double-(His)$_6$-tag, the binding to Ni$^{2+}$-nitrilotriacetic acid-coated substrates was significantly improved. Surface plasmon resonance analysis showed a significantly tighter binding with at least a threefold slower dissociation. The improved binding characteristics thus enabled non-purified probes even in the format of crude expression supernatants to be directly applied thereby eliminating the need for any time-consuming pre-purification step(s) prior to the immobilization. While the double-(His)$_6$-tag probes were found to be expressed equally well as compared to the single-(His)$_6$-tag probes, they displayed better long-term functional on-chip stability. Taken together, the results demonstrate the generic potential of double-(His)$_6$-tag recombinant antibodies for the facile fabrication of high-density antibody microarrays.

Keywords:
Affinity coupling / Affinity tag / Antibody microarray / On-chip purification / Orientated coupling

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Abbreviations: CT, choleratoxin subunit B; His, histidine; NTA, nitrilotriacetic acid; RT, room temperature; scFv, single-chain fragment variable; SAM, self-assembled monolayers; SinFab, single framework antibody fragment; SPR, surface plasmon resonance

1 Introduction

Analogous to DNA microarrays, antibody microarrays offer novel and distinct possibilities to perform rapid, sensitive and high-throughput global proteome analysis [1–3]. The technology will provide new means to perform differential protein expression profiling of healthy vs. diseased samples that will play a key role within disease diagnostics, biomarker discovery and drug target identification [1–5]. High-throughput proteomic analysis may also help us to close the existing information gap between genomics and proteomics [1, 4–6].
Major efforts are currently under way by us [7–11] and others [e.g., 12–16] to develop the antibody microarray technology and the first generation(s) of mainly low- to medium-density microarrays have been successfully designed [1–3, 17, 18]. We have shown that our human recombinant single-chain fragment variable (scFv) antibody library, the n-CoDeR library genetically constructed around one framework [19], was an excellent probe source for array applications [7–11]. Briefly, these single framework antibody fragments (SinFab) were found to display a high functionality on a wide variety of solid supports (with different coupling chemistries) [8, 9, 11, 20], an outstanding long-term functional on-chip stability (up to 16 months) ([8] and Steinhauer, C., Borrebaeck, C. A. K., Wingren, C., submitted for publication), and excellent specificity and sensitivity (pM to fM range) even when targeting complex proteomes [10, 11].

However, in the process of designing high-density antibody microarrays, several critical key (logistical) issues have been identified that remain to be resolved [1, 21]. In particular, there is a great demand for selective and specific immobilization techniques enabling non-purified probes to be directly applied [1, 21]. To this end, affinity coupling offers an attractive approach that will enable crude probe preparations to be purified, coupled, enriched and specifically orientated in a one-step procedure directly on the chip (affinity-on-a-chip) at high density [1, 21]. So far, the selection of functionalized substrates allowing affinity binding of arrayed affinity-tagged probes include Ni2+-slides (http://www.xenopore.com and [20]), streptavidin-coated slides (http://www.xenopore.com and [22–24]), biotin-coated substrates (http://www.zeptosens.com), and DNA-modified slides [25, 26]. Recent work has shown that the performance of antibody (protein) microarrays fabricated by affinity directed immobilization vs. standard coupling (adsorption or covalent coupling) was in many cases superior [22–27]. The most prominent features observed included better spot morphologies, enhanced experimental reproducibility, significantly reduced (at least 100-fold less) amount of probe required, increased functionality and improved sensitivity [22–27].

The specific interaction between genetically engineered single-hexahistidine (His)6-tagged proteins and Ni2+-nitrilotriacetic acid (Ni2+-NTA) has for the last two decades become a powerful and generic tool for protein purification and immobilization [28, 29]. While we and others have recently demonstrated the potential of the set-up for specific probe immobilization in microarray applications [9, 11, 20, 27, 30], the system suffers from low affinity mainly due to its fast dissociation (Kd of about 1 μM) [31]. Interestingly, a consecutive double-(His)6-tag, composed of two single-(His)6-tags separated by a 11-amino acid spacer, was recently designed that may provide improved binding characteristics [32, 33].

In this study, we have engineered double-(His)6-tagged SinFab and compared their binding properties with their single-(His)6-tagged counterparts on different Ni2+-NTA-coated substrates. The results showed that the novel tag design significantly improved the affinity coupling step (tighter binding and slower dissociation) thereby allowing non-purified SinFab to be directly applied. The generic potential of double-(His)6-tagged recombinant SinFab or protein probes in general for the design and facile fabrication of high-density microarrays is demonstrated.

## 2 Materials and methods

### 2.1 Design of pFab5c.2his

The plasmid pTA-His-3 [32], containing a fragment encoding the double-(His)6-tag made up of a single (His)6-tag, a 11-amino acid (SRAWRHPQFGG) linker followed by a second (His)6-tag, was used as starting template. The fragment encoding the double-(His)6-tag was amplified using the primers, 1964-forward: ataaaggcgcgcggctgtgcgtctgtgcgcgttctgcgtgccac and 1945-reverse: ataaaggcgcgcggatagttcctcctttcag, and Taq polymerase (Qiagen, Hilden, Germany) according to manufacturer’s instructions and standard PCR protocol for 30 cycles. The resulting fragment contained a 5’ Not I cleavage site and a 3’ Sac II cleavage-site (underlined). Next, the fragment and the pFab5c.his-gIIII plasmid [34] were cleaved with Not I and Sac II (New England Biolabs, NEB, Beverly, MA, USA) according to the instructions provided by the manufacturer. Subsequently, the gene fragments were assembled in a standard ligation using T4 DNA ligase (NEB). The sequence of the resulting plasmid, pFab5c.2his, was confirmed by sequence analysis (MWG Biotech, Ebersberg, Germany).

### 2.2 Design and production of single- and double-(His)6-tagged CT17 and FITC8

The single-chain Fv (scFv) antibody fragments directed against cholera toxin subunit B (CT) (clone CT-17) and FITC (clone FITC-8) were selected from the human recombinant scFv antibody phage-display n-CoDeR library [19] and kindly provided by BioInvent International AB (Lund, Sweden). These scFv antibodies carried the standard single-(His)6-tag. To generate double-(His)6-tagged scFv, the gene fragment encoding the antibodies were cleaved out of the corresponding pFab5c.his-gIII plasmids using Sfi I (5’) and Not I (3’) (NEB), and subsequently ligated into pFab5c.2his. The sequence of the constructs was confirmed by sequence analysis (MWG Biotech). The antibody containing plasmids were then transformed into RbCl competent Escherichia coli (strain XL 1-Blue).

Both single- and double-(His)6-tagged scFv were produced in E. coli. Soluble scFv molecules, all carrying the C-terminal single-(His)6-tag or double-(His)6-tag, were purified from both the periplasmic space and the expression supernatant by affinity chromatography on Ni2+-NTA (Qiagen). Bound molecules were eluted with 250 mM imidazole (in 50 mM sodium phosphate, 300 mM sodium chloride, pH 8).
Protein Arrays were determined using a MicroBCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL). The post-production yields were evaluated by means of antigen-specific dot blot analysis [8], SDS-PAGE and Western blot. The amount of monomers and dimers (multimers) were measured by HPLC-SE chromatography on Superdex 75 prep grade (Amersham Bioscience, Piscataway, NJ).

2.3 Antigens (CT and FITC) and secondary reagents
CT (Sigma, St. Louis, MO) was labelled with Cy5 monoreactive dye following the recommendations of the manufacturer (Amersham Bioscience). Similarly, streptavidin (Sigma) was labelled with Alexa 546 (Molecular Probes, Eugene, OR) according to manufacturer’s instructions. Excess dye was removed by extensive dialysis against PBS, 0.05% sodiumazide, where after the labelled proteins were stored in the dark at 4°C until further use. Biotinylated CT (CT-Biotin) and FITC (FITC-Biotin) were purchased from Sigma. Streptavidin labelled with horseradish peroxidase (HRP) was obtained from Dako A/S (Glostrup, Denmark).

2.4 Biacore binding assay and data analysis
The binding affinity of single- and double-(His)_{6}-tagged scFv for Ni^{2+}-NTA was evaluated by surface plasmon resonance (SPR) (Biacore, Uppsala, Sweden). Monomeric fractions of the purified scFv molecules were generated by HPLC-SE chromatography on Superdex 75 prep grade (Amersham) just prior to the SPR analysis. Ni^{2+} was immobilized on the Sensor Chip NTA (Biacore) according to the instructions provided by the manufacturer using an HEPES complete (HC) running buffer without EDTA, pH 6.8. The scFv antibodies were injected at a constant flow-rate of 20 μL/min at a concentration of 50, 100, 250 and 500 nM. Each sensorgram was run in duplicates or more. As the data could not be fitted to the standard equations, no affinity constants could readily be determined. Instead, the half-life (T_{1/2}) of the formed complex was estimated.

2.5 Binding assay on XenoSlide N substrates
The binding efficiencies of single- and double-(His)_{6}-tagged scFv were compared on the, to date, only commercially available Ni^{2+}-chelate derivatised substrate, XenoSlide N (Xenopore, Hawthorne, NJ). To this end, four different antibody preparations, including periplasmic preparation, expression-supernatant, 0.1 mg/mL pure scFv spiked in PBS containing 1% w/v fat-free milk powder, and 0.1 mg/mL pure scFv in PBS were generated. The antibody preparations were spotted in 16 x 8 arrays using the non-contact Biochip Arrayer1 (Perkin Elmer Life & Analytical Sciences, Boston, MA). The scFv were arrayed as single droplets (about 333 pL) at a spot-to-spot distance of 200 μm. The arrays were blocked with 5% w/v fat-free milk powder, 0.5% w/v Tween-20 in PBS for 1 h. All incubations were performed in a humidity chamber at room temperature (RT). Subsequently, the arrays were washed four times with 0.5% v/v Tween-20 in PBS (PBS-T), and incubated with 1 μg/mL CT-Cy5 or 40 ng/mL FITC-Biotin in 1% w/v fat-free milk powder, 0.5% v/v Tween-20 in PBS for 1 h. The FITC-array was washed four times in PBS-T, and incubated with 20 μg/mL streptavidin-Alexa 546 in 1% w/v fat-free milk powder, 0.5% v/v Tween-20 in PBS for another 1 h. Finally, the arrays were washed in PBS-T, allowed to dry out and immediately scanned using the confocal ScanArray Express microarray scanner (Perkin Elmer Life & Analytical Sciences). The ScanArray Express software V2.0 (Perkin Elmer Life & Analytical Sciences) was used to quantitate the intensity of each spot, using the fixed circle method. Each data point represents the mean value of eight replicates after subtracting local background and negative control (nonspecific scFv). CT-17 was used as negative control on the FITC-8 arrays and vice versa.

2.6 Long-term functional on-chip stability on XenoSlide N substrates
The long-term functional on-chip stability of single- and double-(His)_{6}-tagged scFv molecules was determined on the XenoSlide N glass slides (Xenopore). The freshly prepared scFv were arrayed at three different concentrations, 1 mg/mL (11 femtomol/spot), 0.5 mg/mL (5.5 fmol/spot) and 0.1 mg/mL (1.1 fmol/spot). The microarrays were fabricated and developed as described above. Of note, all seven slides were fabricated on day 0, allowed to dry out and stored in a dark microscope slide box at RT, before the slides were analysed at different time points (days 0, 6, 13, 45, 97, 122 and 151).

2.7 Binding assay on Ni^{2+}-NTA HisSorb strips
The binding efficiencies of single-(His)_{6}-tagged and double-(His)_{6}-tagged scFv antibodies on Ni^{2+}-NTA-coated HisSorb strips (Qiagen) were determined for different scFv formats, including periplasmic preparation, crude expression supernatant, 0.1 mg/mL pure scFv spiked in 1% w/v fat-free milk powder, PBS and 0.1 mg/mL pure scFv in PBS. Of each scFv preparation, 200 μL was applied in triplicates and allowed to couple at 4°C overnight. Next, the wells were washed four times with PBS-T where after the strips were blocked with 5% w/v fat-free milk powder, 0.5% v/v Tween-20 in PBS at 1 h at RT. After washing in PBS-T, the strips were incubated with 1 μg/mL CT-Biotin or 40 ng/mL FITC-Biotin in 1% w/v fat-free milk powder, 0.5% v/v Tween-20 in PBS for 1 h at RT. The strips were washed in PBS-T and incubated with streptavidin-HRP diluted 1:1000 in 1% w/v fat-free milk powder, 0.5% v/v Tween-20 in PBS for 1 h at RT. Finally, the strips were washed in PBS-T whereafter any signals were
detected at 490 nm using the o-phenylenediamine (OPD) detection system (Sigma). Each data point represents the mean value of three replicates after subtracting negative control (non-specific scFv). CT-17 was used as negative control for FITC-8 and vice versa.

3 Results

3.1 General remarks

The development of specific and selective immobilization techniques enabling non-purified probes to be directly utilized, will be a key step when designing the next generation(s) of antibody microarray technology platforms compatible with high-density layouts. To optimize the affinity coupling of our human recombinant SinFab, designed for microarray applications, to Ni\textsuperscript{2+}-NTA-coated substrates, we have re-engineered their affinity tag by replacing the standard single-(His)\textsubscript{6}-tag with a novel consecutive double-(His)\textsubscript{6}-tag. To this end, two SinFab, directed against CT (clone CT-17) and FITC (clone FITC-8), were selected as model probes. The probe-tag designs were evaluated with respect to their binding properties (affinity) and other critical biophysical properties (e.g. productivity, on-chip stability etc).

3.2 Key biophysical properties

Double-(His)\textsubscript{6}-tagged variants of both CT-17 and FITC-8 were successfully engineered. In Fig. 1, selected key biophysical properties of the single- and double-(His)\textsubscript{6}-tagged

![Figure 1](image-url)
clones are compared. The results show that the single- and double-(His)$_6$-tagged variants of both CT-17 and FITC-8 were expressed in the low $\mu$g/mL range, indicating that the tag-design did not impair the protein expression levels (Fig. 1A). Similarly, the tag-design did not significantly affect the content of monomers vs. multimers as measured by HPLC-SE analysis of affinity purified probe preparations (Fig. 1B).

To evaluate the long-term functional stability of the arrayed probes, the activity of the antibodies, which were all spotted (day 0) and dried before storage, was determined as a function over time (Fig. 1C–F). While both tag-variants of each SinFab were still active after 151 days, the double-(His)$_6$-tagged clones displayed 2.3 (93 vs. 40%, CT-17) to 29 (FITC-8) times higher activity (Fig. 1C and E). The activity profiles of each matching SinFab pair changed over time, but in a similar manner, following a parabolic-like shaped curve with a maximum between days 50 and 100 (Fig. 1C and E). Furthermore, the observed increase in activity was 2 (CT-17) (Fig. 1D) to 50 (FITC-8) (Fig. 1F) times higher for the double-(His)$_6$-tagged clones than for their single-(His)$_6$-tagged counterparts. Of note, the results also showed that the relative increase in activity of the double-(His)$_6$-tagged SinFab occurred early and within a narrow range of time after array fabrication (between days 0 and 13), where after the relative activity quota was almost constant (Fig. 1D and F). Similar results were obtained irrespective of the amount of SinFab spotted (1.1 to 11 femtomol/spot) (Fig. 1G and data not shown). As illustrated in Fig. 2G for the double-(His)$_6$-tag FITC-8, the spot morphology was consistent or improved over time.

Taken together, the results showed that replacing the single-(His)$_6$-tag with a consecutive double-(His)$_6$-tag improved the long-term functional on-chip stability of arrayed SinFab.

### 3.3 Binding assay on Ni$^{2+}$-NTA chips using SPR

In order to compare the affinities of single- and double-(His)$_6$-tagged SinFab molecules for Ni$^{2+}$-NTA, monomeric fractions of all variants were analysed on Biacores Ni$^{2+}$-NTA sensor chip. Figure 2A and D shows the binding curves of single- and double-(His)$_6$-tagged CT-17 and FITC-8 at 100 and 500 nM concentrations, respectively. In both cases, the SPR results showed that the dissociation of the double-(His)$_6$-tagged SinFab was slower and that significantly more protein remained bound after buffer wash. Figure 2B and E shows an overlay plot of the dissociation curves for single- and double-(His)$_6$-tagged probes at 500 nM, where two to three times more double-(His)$_6$-tagged proteins remained bound after buffer wash, again indicating on significantly stronger binding. As the data could not be fitted to the standard equations, affinity constants could not be determined. Instead, the half-life ($T_{1/2}$) of the formed complex was estimated (Fig. 2C and F). In the case of both CT-17 (Fig. 2C) and FITC-8 (Fig. 2F), the results showed that the $T_{1/2}$ was significantly (two to four times) longer for the double-(His)$_6$-tagged probes than for their single-(His)$_6$-tagged counterparts. Moreover, the $T_{1/2}$ increased significantly (two to three times) with decreasing SinFab concentration, indicating the repetitive binding-dissociation events taking place as the number of freely available Ni$^{2+}$-NTA groups increased. The latter feature was predominantly observed only for the double-(His)$_6$-tagged variants. Taken together, the data showed that the double-(His)$_6$-tagged SinFab displayed a significantly tighter binding to Ni$^{2+}$-NTA with at least a threefold slower dissociation as compared to their single-(His)$_6$-tagged counterparts.

**Figure 2.** Comparison of the binding properties of two single- versus double-his-tagged SinFab molecules (clones CT-17 and FITC-8) determined by surface plasmon resonance analysis on Ni$^{2+}$-derivatised Biacore chips. (A) Biacore sensorgrams for CT-17-his (i – 500 nM, ii – 100 nM) and CT-17-2his (iii – 500 nM, iv – 100 nM). (B) Biacore sensorgrams showing the relative dissociation response of CT-17-his (i – 500 nM) and CT-17-2his (ii – 500 nM). (C) The half-life, $T_{1/2}$, of bound CT-17-his and CT-17-2his. (D) Biacore sensorgrams for FITC-8-his (i – 500 nM, ii – 100 nM) and FITC-8-2his (iii – 500 nM, iv – 100 nM). (E) Biacore sensorgrams showing the relative dissociation response of FITC-8-his (i – 500 nM) and FITC-8-2his (iii – 500 nM). (F) The half-life, $T_{1/2}$, of bound FITC-8-his and FITC-8-2his.
3.4 Binding assay on Ni\textsuperscript{2+}-NTA coated XenoSlide N substrates

Next, we compared the immobilization of single- and double-(His)\textsubscript{6}-tagged SinFab molecules to XenoSlide N, a Ni\textsuperscript{2+}-NTA functionalized glass slide, especially designed for protein microarray applications (Fig. 3). To this end, four different probe preparations of each SinFab design were applied, including 100 \(\mu\text{g/mL}\) pure SinFab, 100 \(\mu\text{g/mL}\) SinFab spiked into PBS containing 1\% v/w fat-free milk powder, periplasmic SinFab preparation (\(\leq 150 \mu\text{g/mL}\)) and crude SinFab expression supernatant (\(\sim 4 \mu\text{g/mL}\)).

In the case of CT-17, specific signals could only be detected for pure probes, with the double-(His)\textsubscript{6}-tagged variant displaying the highest signal intensity and the best spot morphology (Fig. 3A). In the case of FITC-8, specific signals could be detected for all probe formats (Fig. 3B). Notably, in all four cases, the double-(His)\textsubscript{6}-tagged SinFab displayed higher signal intensities and equal or better spot morphologies than the single-(His)\textsubscript{6}-tagged clone, clearly indicating the increased assay sensitivity. In addition, it also demonstrated that non-purified probes could be directly applied. Hence, the results showed that substituting the single-(His)\textsubscript{6}-tag with a double-(His)\textsubscript{6}-tag improved the coupling of SinFab to Ni\textsuperscript{2+}-NTA-coated XenoSlide N substrates by either (i) simply increasing the amount of specific probes immobilized, (ii) improving the functionality of the coupled probes, and/or (iii) generating more homogeneous spots.

![Figure 3. Comparison of the binding efficiencies of two single- vs. double-his-tagged SinFab molecules (clones CT-17 and FITC-8) to Ni\textsuperscript{2+}-coated XenoSlide N glass slides. (A) CT-17-his – white bars, CT-17-2his – grey bars. (B) FITC-8-his – white bars, FITC-8-2his – grey bars. The SinFab were applied as crude periplasmic preparations, crude expression supernatants, spiked (0.1 mg/mL) into 1\% (w/v) fat-free milk powder in PBS, and as pure preparations (0.1 mg/mL in PBS). Relative signal intensities of adsorbed and functionally active SinFab molecules are shown.](image)

3.5 Binding assay on Ni\textsuperscript{2+}-NTA HisSorb strips

Finally, we compared the coupling efficiency of single- and double-(His)\textsubscript{6}-tagged SinFab to HisSorb strips, Ni\textsuperscript{2+}-NTA functionalized plastic strips, especially designed for immobilization of his-tagged proteins from complex mixtures (Fig. 4). Again (Figs. 3 and 4), four different probe preparations of each SinFab design were applied, including 100 \(\mu\text{g/mL}\) pure SinFab, 100 \(\mu\text{g/mL}\) SinFab spiked into PBS containing 1\% v/w fat-free milk powder, periplasmic SinFab preparation (\(\leq 150 \mu\text{g/mL}\)) and crude SinFab expression supernatant (\(\sim 4 \mu\text{g/mL}\)).

Figure 4A shows the immobilization of single- and double-(His)\textsubscript{6}-tagged CT-17. In all four cases, significantly stronger (2 to 8 times) signal intensities were observed for the immobilized double-(His)\textsubscript{6}-tagged variant than for the single-(His)\textsubscript{6}-tagged clone. Similar results were observed for FITC-8, although the observed differences in signal intensities for the double- and single-(His)\textsubscript{6}-tagged SinFab were even more pronounced (2 to 100 times) (Fig. 4B). In fact, the double-(His)\textsubscript{6}-tag design was in all cases critical for observing any signal intensities at all, clearly indicating increased assay sensitivity. Hence, the results showed that the double-(His)\textsubscript{6}-tag design significantly improved the immobilization of SinFab to Ni\textsuperscript{2+}-NTA coated HisSorb strips by (i) improving the functionality of the coupled probes and/or (ii) by simply increasing the amount of SinFab immobilized. Taken together, by adopting the double-(His)\textsubscript{6}-tag design, we have

![Figure 4. Comparison of the binding efficiencies of two single- vs. double-his-tagged SinFab molecules (clones CT-17 and FITC-8) to Ni\textsuperscript{2+}-NTA HisSorb Strips. (A) CT-17-his – white bars, CT-17-2his – grey bars. (B) FITC-8-his – white bars, FITC-8-2his – grey bars. The SinFab were applied as crude periplasmic preparations, crude expression supernatants, spiked (0.1 mg/mL) into 1\% (w/v) fat-free milk powder in PBS, and as pure preparations (0.1 mg/mL in PBS). Relative signal intensities of adsorbed and functionally active SinFab-molecules are shown.](image)
developed a set-up allowing non-purified probes to be directly applied, thereby eliminating the need for any time-consuming pre-purification steps.

4 Discussion

In this study, we have shown that the affinity coupling of SinFab, designed for microarray applications, to Ni\(^{2+}\)-NTA-coated substrates could be significantly improved by replacing their standard single-(His)\(_6\)-tag with a novel consecutive double-(His)\(_6\)-tag. The specific interaction between engineered (His)\(_6\)-tagged proteins and Ni\(^{2+}\)-NTA have been commonly used for years to purify and immobilize proteins [28, 29], and more recently also within protein microarray applications for specific and selective affinity coupling of probes [9, 11, 20, 27, 30]. In the latter case, Ni\(^{2+}\)-NTA-coated glass slides (XenoSlide N) [http://www.xenopore.com and [9, 27]), Ni\(^{2+}\)-NTA modified lipid surfaces [20, 30], multivalent NTA surfaces based on self-assembled monolayers (SAM) [35], and anti-(His)\(_6\)-tag antibody coated slides [11] have so far been successfully used. Despite the proven potential, the system suffers from low affinity, mainly due to fast dissociation [31], which may impair the use of single-(His)\(_6\)-tagged probes [30].

Here, we demonstrated that the binding to Ni\(^{2+}\)-NTA could be significantly improved by introducing the consecutive double-(His)\(_6\)-tag [32, 33], leading to a tighter binding and more than threefold slower dissociation (Fig. 2). Previously, it has been shown that simply prolonging the single (His)\(_6\)-tag to ten consecutive histidine residues was an alternative route of improving the affinity [36]. However, such a design was also found to lower the expression yields and increasing the oligomerization state of the purified protein, which often lead to loss of function [36]. In our case, the introduction of the double-(His)\(_6\)-tag did neither negatively affect the expression yields (Fig. 1A) nor the oligomerization state of the purified SinFab (Fig. 1B). Even higher relative activities/signal intensities were obtained for the double-(His)\(_6\)-tagged SinFab than for the single-(His)\(_6\)-tagged counterparts including the his-tag–Ni-NTA set-up, have shown that affinity coupling often lead to an increased functionality of the immobilized probes. (ii) that a higher amount of probes had been coupled, or (iii) a combination thereof remains to be elucidated. Notably, other studies using a variety of systems, including the his-tag–Ni-NTA set-up, have shown that affinity coupling often lead to an increased functionality of the immobilized probes [22–27].

However, the addition of several histidine residues (up to ten) has been shown to decrease the stability and solubility of the tagged protein [37]. Similarly, our results indicated that the double-(His)\(_6\)-tagged probes were less stable in solution than the corresponding single-(His)\(_6\)-tagged variants (data not shown). In contrast, our data clearly showed that the long-term functional on-chip stability of the arrayed double-(His)\(_6\)-tagged SinFab was better than for the single-(His)\(_6\)-tagged SinFab (Fig. 1C–F). In agreement with these observations, we have recently demonstrated the lack of correlation between the on-chip functional stability of a set of scFv antibodies, including some of our SinFab, and their thermal stability in solution (as measured by differential scanning calorimetry) [8].

Previously, we had shown that fabricated SinFab microarrays could be stored dry at RT for about 50 days while still displaying high activity (>70%) [8]. In this study, the SinFab were found to be highly active even after 151 days of storage (Fig. 1C–F). Of note, recent data have implied that the time of storage could be extended up to 16 months while still maintaining adequate activity (Steinhauer, C., Borrebaeck, C. A. K., Wingren, C., submitted for publication). To the best of our knowledge, these are so far the best on-chip functional stabilities reported for arrayed recombinant scFv antibody fragments. Interestingly, the activity profiles of the spotted SinFab changed over time, following a parabolic-like shaped curve (Fig. 1C and E). The initial increase in activity over time is a phenomenon commonly observed also for intact antibodies on a wide variety of solid supports [38, 39]. The reason(s) for this is still not clear, but could at least partly be explained by probes that denatured upon deposition onto the solid support have refolded and thereby regained their functional activity (Steinhauer, C., Borrebaeck, C. A. K., Wingren, C., submitted for publication).

Adopting an opposite approach, and addressing the NTA-substrate layout instead of the tag-design to improve the (His)\(_6\)-tag-NTA interaction has also proven fruitful [35, 40]. Simply replacing the Ni\(^{2+}\)-ions with other divalent ions, such as Cu\(^{2+}\), increase the binding strength [40, 41]. Recently, Tinzali et al. [35] designed a multivalent NTA substrate based on SAM. To this end, his-NTA was coupled through an oligoethylene glycol to alkyl thiols to generate a novel, multivalent compound for formation of mixed SAM on gold. These multivalent chelator surfaces were found to significantly improve the stability of bound single-(His)\(_6\)-tagged proteins [35]. It was concluded that stable binding of single-(His)\(_6\)-tagged proteins took place at a specific surface threshold value of NTA lipid [35]. In other words, cumulated NTA clusters with multivalent interaction sites to single-(His)\(_6\)-tags may overcome the limitation of protein dissociation in the conventional single-(His)\(_6\)-tag-NTA interaction. Interestingly, our SPR analysis showed that the T\(_{1/2}\) of the single-(His)\(_6\)-tag-NTA interaction increased (<twice) when the ratio between available NTA to injected amount of SinFab was increased (up to ten times), indicating on similar effects (Fig. 1C and F). Most importantly, these effects were even more pronounced in the case of the double-(His)\(_6\)-tagged SinFab (Fig. 1C and F), further highlighting the importance of the tag-design.

It should be noted that the design of high-performing NTA-functionalized substrates are much sought for. The Ni\(^{2+}\)-NTA functionalized glass slides (XenoSlide N) [http://www.xenopore.com] used in this study are among the few, if not the only, commercially available NTA modified solid support suitable for microarrays. Although performing well,
especially when using pure probes [11, 27], we have recently observed that the presence of high amount of irrelevant proteins impaired the specific immobilization of his-tagged probes on the Ni$^{2+}$-coated slides [27]. These problems might, at least partly, be addressed by adopting the double-(His)$_6$-tag design presented in this report (Fig. 3).

To date, a majority of all antibody (and protein) microarrays have been based on pre-purified probes [1, 3, 15, 17, 18]. This has been practically and logistically feasible, as mainly low-to-medium density microarrays have been fabricated [12, 13]. However, the logistics behind designing the design presented in this report (Fig. 3).

In summary, we have extended previous findings and shown that the improved binding properties of the double-(His)$_6$-tagged SinFab for Ni$^{2+}$-NTA readily enabled non-purified probes even in the format of crude expression supernatants to be readily applied onto Ni$^{2+}$-NTA functionalized substrates. Taken together, we have outlined the generic potential of double-(His)$_6$-tagged recombinant SinFab or protein probes in general for the design and facile fabrication of high-density microarrays.

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5 References


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