



# Alkaline phosphatase-fused reobody as a new format of immuno-reagent for an immunoassay



Hyo-Deok Seo <sup>a</sup>, Joong-jae Lee <sup>a</sup>, Yu Jung Kim <sup>c</sup>, Oliver Hantschel <sup>b</sup>, Seung-Goo Lee <sup>c</sup>, Hak-Sung Kim <sup>a,\*</sup>

<sup>a</sup> Department of Biological Sciences, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon, 305-701, Republic of Korea

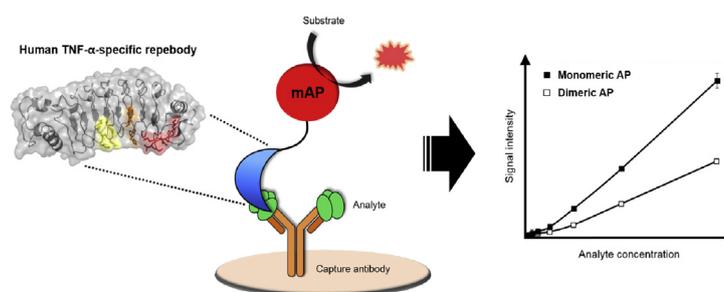
<sup>b</sup> School of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

<sup>c</sup> Industrial Biotechnology and Bioenergy Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Republic of Korea

## HIGHLIGHTS

- A human TNF- $\alpha$  (hTNF- $\alpha$ )-specific reobody was selected using a phage display.
- A monomeric alkaline phosphatase (mAP) was genetically fused to the reobody.
- mAP-fused reobody enabled detection of hTNF- $\alpha$  with high sensitivity and accuracy.
- mAP-fused reobody can be widely used as a new immuno-reagent in immunoassays.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

### Article history:

Received 2 August 2016

Received in revised form

7 November 2016

Accepted 9 November 2016

Available online 12 November 2016

### Keywords:

Monomeric alkaline phosphatase

Reobody

Genetic fusion

Immuno-reagent

Immunoassay

## ABSTRACT

Enzyme-linked immunoassays based on an antibody-antigen interaction are widely used in biological and medical sciences. However, the conjugation of an enzyme to antibodies needs an additional chemical process, usually resulting in randomly cross-linked molecules and a loss of the binding affinity and enzyme activity. Herein, we present the development of an alkaline phosphatase-fused reobody as a new format of immuno-reagent for immunoassays. A reobody specifically binding to human TNF- $\alpha$  (hTNF- $\alpha$ ) was selected through a phage display, and its binding affinity was increased up to 49 nM using a modular engineering approach. A monomeric alkaline phosphatase (mAP), which was previously isolated from a metagenome library, was genetically fused to the reobody as a signal generator, and the resulting reobody-mAP fusion protein was used for direct and sandwich immunoassays of hTNF- $\alpha$ . We demonstrate the utility and potential of the reobody-mAP fusion protein as an immuno-reagent by showing the sensitivity of 216 pg mL<sup>-1</sup> for hTNF- $\alpha$  in a sandwich immunoassay. Furthermore, this reobody-mAP fusion protein enabled the detection of hTNF- $\alpha$  spiked in a serum-supplemented medium with high accuracy and reproducibility. It is thus expected that a mAP-fused reobody can be broadly used as an immuno-reagent in immunoassays.

© 2016 Elsevier B.V. All rights reserved.

## 1. Introduction

An enzyme-linked immunosorbent assay (ELISA) based on an antibody-antigen interaction is widely used to detect diverse

\* Corresponding author.

E-mail address: [hskim76@kaist.ac.kr](mailto:hskim76@kaist.ac.kr) (H.-S. Kim).

analytes owing to its simplicity, sensitivity, and selectivity. It usually requires a primary capture antibody and a secondary detection antibody labeled with enzymes, such as horseradish peroxidase (HRP) and alkaline phosphatase (AP) [1]. The conjugation of HRP to an antibody or other protein binders is usually carried out through a random chemical reaction, resulting in unstable and randomly cross-linked molecules [2]. A random chemical approach might cause a loss in the binding affinity of the protein binders and the enzyme activity, and it is hard to control the conjugation stoichiometry. Furthermore, the production cost and variation in the HRP activity hamper its broad use in immunoassays [3]. To overcome such drawbacks, the genetic fusion of an alkaline phosphatase to an alternative protein binder, such as a single-chain variable fragment (scFv) and the variable domain of a heavy chain antibody (VHH, nanobody) from a camelid, has been recently attempted [4–7]. Among numerous types of AP isolated from diverse sources, dimeric AP (dAP) from *E. coli* has been most intensively studied, and is currently used for the labeling of protein binders through genetic fusion. It was reported that dAP is more stable and accurate than HRP for an immunoassay [8]. Despite such favorable features of dAP, however, the genetic fusion of a large-sized dAP was revealed to cause a steric hindrance to the target binding owing to the formation of a dimeric structure, which affects the specificity and sensitivity in immunoassays [9,10]. Therefore, AP with a monomeric structure and high catalytic activity is preferred to avoid shortcomings resulting from the conjugation of a dimeric AP. A monomeric AP was recently isolated from a metagenome library, and its biochemical property was characterized [11]. mAP was shown to be expressed at a high level in *E. coli*, displaying a much higher catalytic activity than dAP from *E. coli*, despite its relatively low thermostability.

We previously developed a rebody scaffold composed of Leucine-rich repeat (LRR) modules. The rebody scaffold was shown to be expressed at a high level in *E. coli*, exhibiting high stability against heat, pH, and proteolytic digestion [12–17]. Furthermore, a target-specific rebody is easy to select through a phage display, and its binding affinity can be increased up to a low nanomolar range using a modular engineering approach. Herein, we present the development of a monomeric alkaline phosphatase-fused rebody as a new format of immuno-reagent for an immunoassay. Based on the significance in the diagnosis and monitoring of drug responses for various autoimmune diseases such as rheumatoid arthritis and lupus [18,19], human TNF- $\alpha$  (hTNF- $\alpha$ ) was employed as a model target analyte in this work. We selected a rebody that binds specifically to hTNF- $\alpha$  through a phage display, and increased its binding affinity to 49 nM through modular engineering. mAP isolated from a metagenome library was genetically fused to the rebody as a signal generator, and the resulting rebody-mAP fusion protein was used for an immunoassay of hTNF- $\alpha$ . The performance of the mAP-fused rebody was evaluated in terms of sensitivity and detection limit through both direct and sandwich immunoassays. Details are reported herein.

## 2. Materials and methods

### 2.1. Selection and affinity maturation of anti-hTNF- $\alpha$ rebodies

Rebodies specifically binding to human TNF- $\alpha$  were selected through a phage display as described in our previous work [16]. Briefly, a rebody library was constructed by introducing random mutations on six variable sites of two LRR modules (LRRV2 and LRRV3) of the rebody scaffold and subjected to a phage display selection. hTNF- $\alpha$  in PBS was coated onto an immunotube (Greiner) through incubation at 4 °C overnight. The tube was blocked with 5 mL of a blocking buffer (PBS, 0.05% Tween-20, 1% BSA) for 2 h at

4 °C followed by the addition of a 1 mL phage solution ( $1.0 \times 10^{12}$  cfu mL<sup>-1</sup>). Phages bound to hTNF- $\alpha$  were eluted through 1 mL of a 0.2 M Glycine-HCl solution (pH 2.2), and the eluted phage solution was neutralized with 100  $\mu$ l of 1 M Tris-HCl (pH 9.0) and used to infect 10 mL of *E. coli* XL-1Blue F' cells. After shaking for 1 h at 37 °C, cells were collected and spread onto a 2xYT agar plate containing 100  $\mu$ g mL<sup>-1</sup> of ampicillin, 25  $\mu$ g mL<sup>-1</sup> of tetracycline, and 1% glucose. The cells were grown overnight at 30 °C and incubated again in 2xYT/ATG until the absorbance at 600 nm reached 0.4. The cells were rescued by infection with a VCS M13 helper phage (Stratagene) and further grown overnight at 30 °C with 2xYT/AKT. The cells were removed through centrifugation, and a phage solution was precipitated for 1 h at 4 °C with an ice-cold solution of 20% PEG/NaCl. The precipitated phages were then collected through centrifugation and used for a repeating round of panning. After six rounds of panning, enrichment of the phage particles specifically binding to hTNF- $\alpha$  were identified through a phage immunoassay. For affinity maturation, a library was constructed by randomizing an additional module (LRRV5) or the C-terminal loop region of the selected rebody, and subjected to a phage display selection according to the above procedure.

### 2.2. Protein expression and purification

Human TNF- $\alpha$  (residue 1–157) was cloned into NcoI-XhoI of a pET32a vector (Novagen), and its biological activity was measured using a dual-luciferase assay system [20]. Rebodies were cloned into NdeI-XhoI of the pET21a vector. For the construction of a rebody-AP fusion protein, a rebody gene was cloned into NdeI-BamHI, and either monomeric AP (mAP; residue 61–617) or dimeric AP (dAP; residue 22–450) was subsequently cloned into BamHI-XhoI of the pET21a vector. The AP-fused rebody gene was transformed and expressed in Origami-B *E. coli* cells (Merck Bioscience) to enhance their disulfide bond formation. The transformed cells were grown in an LB medium at 37 °C followed by the addition of IPTG at a final concentration of 0.5 mM for induction. After induction, the cells were incubated at 18 °C for 16 h, harvested through centrifugation at 6500 rpm, and suspended in a lysis buffer (50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10 mM imidazole). The suspended cells were disrupted by sonication followed by centrifugation at 16,000 rpm for 1 h, and the supernatant was collected and purified using a Ni-NTA Superflow column (Qiagen). Soluble proteins were applied to the resin-packed column followed by washing with a buffer (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 30 mM imidazole), and resin-bound proteins were eluted using an elution buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 200 mM imidazole). To remove the thioredoxin tag, eluted hTNF- $\alpha$  was digested using bovin alpha-thrombin (HTI) at 4 °C for 16 h, and then loaded onto a Superdex75 (GE healthcare) column in a PBS buffer (pH 7.4).

### 2.3. Activity assay of recombinant hTNF- $\alpha$

Human embryonic kidney 293T (HEK 293T, ATCC) was cultured in DMEM containing 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic, 200  $\mu$ g mL<sup>-1</sup> geneticin, and normocin at 37 °C in humidified air with 5% CO<sub>2</sub>. All materials for the cell culture were obtained from Invitrogen. Recombinant hTNF- $\alpha$  was purchased from R&D systems, and pNiFty-Lu, a pRL-TK vector, and a dual-luciferase<sup>®</sup> reporter assay system were purchased from Promega. HEK 293T cells ( $1.5 \times 10^5$  well<sup>-1</sup>) were cultured in a 24-well plate for 24 h. The NF- $\kappa$ B-induced luciferase vector (pNiFty-Lu, 0.2  $\mu$ g well<sup>-1</sup>) and internal control luciferase vector (pRL-TK, 0.1  $\mu$ g well<sup>-1</sup>) were co-transfected using lipofectamine 2000 (Invitrogen) in cells and incubated for 4 h. After incubation, the medium was

changed to DMEM containing 10% fetal bovine serum and incubated for 20 h. Commercial sources of hTNF- $\alpha$  (10 ng mL<sup>-1</sup>), hTNF- $\alpha$  (10 ng mL<sup>-1</sup>) produced by *E. coli*, and negative control (human interleukin-6 produced by *E. coli*, 10 ng mL<sup>-1</sup>) were added to each cell and incubated for 8 h. Following stimulation with NF- $\kappa$ B, the relative luciferase activity was determined through a dual-luciferase reporter assay system according to the manufacturer's instructions. All of the experimental values were derived from the averages of at least three separate experiments.

#### 2.4. Competitive immunoassay

hTNF- $\alpha$  was coated directly onto a clear MaxiSorp plate. The rebody-displaying phage particles were incubated at different concentrations of Etanercept ranging from 1 to 100  $\mu$ g mL<sup>-1</sup> (Enbrel, a soluble recombinant receptor of hTNF- $\alpha$ ) as a competitor for 1 h at room temperature, followed by addition onto a clear MaxiSorp plate with coated hTNF- $\alpha$ . After washing the wells five times with PBST, an HRP-conjugated anti-M13 monoclonal antibody (GE healthcare) was added. The absorbance at 450 nm was measured using an infinite M200 plate reader after the addition of 100  $\mu$ l of 1 N H<sub>2</sub>SO<sub>4</sub>.

#### 2.5. Isothermal titration calorimetry

All experiments were carried out using a MicroCal iTC200 (Malvern) at 25 °C. All proteins were prepared in a buffer containing PBS (pH 7.4) after purification using size exclusion chromatography. Typically, 0.2 mM of the rebody was titrated with 0.02 mM of hTNF- $\alpha$ . Titrations were preceded by an initial injection of 0.5  $\mu$ l, and carried out using 2  $\mu$ l injections. The time between injections was 180 s. The data were analyzed using the Origin program (OriginLab), and fitted to a one-site binding model.

#### 2.6. Direct immunoassay

Serially diluted hTNF- $\alpha$  was coated directly onto both a clear MaxiSorp plate and a black MaxiSorp plate (Nunc). After overnight incubation, each well was washed three times with 200  $\mu$ l of a TBS buffer (50 mM Tris (pH 8.0), 150 mM NaCl) and blocked with 200  $\mu$ l of a blocking buffer (ProNa™ Blocking solution) for 2 h at room temperature. The wells were washed five times with a TBST buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.05% tween 20) followed by the addition of 2  $\mu$ g mL<sup>-1</sup> of a mAP-fused rebody and substrate for a signal generation. For a colorimetric assay, 100  $\mu$ l of a solution containing 1 mM *p*-nitrophenyl phosphate (pNPP) (Sigma), 1 M diethanolamine and 2.5 mM CaCl<sub>2</sub> at pH 9.6 was added to each well. The plate was incubated at 30 °C for 2 h, and reaction was stopped with 100  $\mu$ l of 1 M NaOH. The absorbance at 405 nm was measured using an infinite M200 plate reader. For a fluorescent assay, a substrate buffer solution (1 M diethanolamine, 2.5 mM CaCl<sub>2</sub>, pH 9.6) containing 1 mM 4-Methylumbelliferyl phosphate (4-MUP) was added to black plates. After incubation for 2 h at 30 °C, the reaction was stopped with 100  $\mu$ l of a 1 M K<sub>2</sub>HPO<sub>4</sub>-KOH buffer (pH 10.4). The fluorescence intensity was measured through excitation at 320 nm and emission at 460 nm using an infinite M200 plate reader.

#### 2.7. Sandwich immunoassay

An anti-human hTNF- $\alpha$  antibody (Mab1, Biologend) was used as a primary capture antibody for a sandwich immunoassay. A black MaxiSorp plate was coated overnight with 100  $\mu$ l of Mab1 (2  $\mu$ g mL<sup>-1</sup>). The plate was blocked with a 200  $\mu$ l blocking buffer (ProNa™ Blocking solution) for 2 h at room temperature followed

by the addition of serially diluted hTNF- $\alpha$ . After incubation at room temperature for 1 h, the plate was washed five times with a TBST buffer, and 2  $\mu$ g mL<sup>-1</sup> of a monomeric or dimeric alkaline phosphatase-fused rebody (rB3-mAP or rB3-dAP) was added to each well. For a fluorescent assay, a substrate buffer solution (1 M diethanolamine, 2.5 mM CaCl<sub>2</sub> for rB3-mAP, 2.5 mM MgCl<sub>2</sub> for rB3-dAP, pH 9.6) containing 1 mM 4-Methylumbelliferyl phosphate (4-MUP) was added to the black plates. The signal intensity was measured using an infinite M200 plate reader. The limit of detection (LOD) of a direct immunoassay and a sandwich immunoassay was calculated based on the mean concentration of the blank plus three standard deviations of the blank. All experiments were carried out in triplicate.

#### 2.8. Validation test

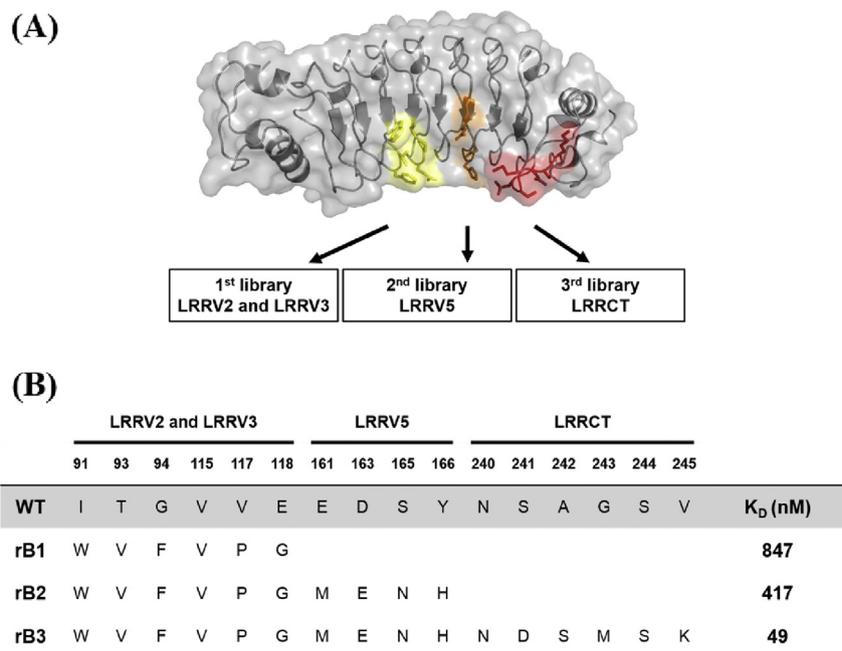
Different concentrations of hTNF- $\alpha$  (0.63, 1.25, 2.5, and 5 ng mL<sup>-1</sup>) were spiked in a Roswell Park Memorial Institute (RPMI) cell culture medium supplemented with a 10% fetal bovine serum (Hyclone™). The concentration of hTNF- $\alpha$  in the medium was determined through a sandwich immunoassay using a mAP-rebody. A total of 10 ng mL<sup>-1</sup> of hTNF- $\alpha$  was used to adjust the difference in signal intensity between the standard curve and spiked hTNF- $\alpha$ . The coefficient of variation (%CV) was calculated as  $CV = \frac{\sigma}{\mu} * 100\%$  ( $\sigma$ , standard deviation;  $\mu$ , mean).

### 3. Results and discussion

#### 3.1. Selection and affinity maturation of anti-human TNF- $\alpha$ rebody

Prior to the selection of rebody against hTNF- $\alpha$ , we cloned and expressed hTNF- $\alpha$  as a soluble form in *E. coli*. Recombinant hTNF- $\alpha$  was shown to have a homotrimeric structure through gel permeation chromatography (Fig. S1A). We checked biological activity of recombinant hTNF- $\alpha$  using a dual-luciferase assay system (Fig. S1B). This cell-based assay system was revealed to be simple and effective for analyzing the biological activity and functionality of hTNF- $\alpha$  [21]. The cell-based assay system revealed that recombinant hTNF- $\alpha$  maintained its biological activity (Fig. S1C).

To select rebody that specifically bind to hTNF- $\alpha$ , a rebody library (2  $\times$  10<sup>8</sup> clones) was constructed by introducing random mutations at six variable sites of two LRR modules (LRRV2 and LRRV3) and subjected to a phage display selection (Fig. 1A). After six rounds of a panning against hTNF- $\alpha$ , 62 clones were selected based on their signal intensity (Fig. S2A). To obtain insight into the binding epitope, we conducted a competitive phage immunoassay for the primarily selected clones using Etanercept as a competitor. Etanercept is a Fc-fused human TNF- $\alpha$  receptor 2, and is clinically used for the treatment of rheumatoid arthritis [22]. As a result, 7 clones showing significantly decreased signal intensity in the presence of Etanercept were selected (Fig. S2B). The selected clones were expressed in *E. coli*, and their binding affinities for hTNF- $\alpha$  were determined through isothermal titration calorimetry (ITC) (Fig. S3A). Of them, rB1 showing the highest binding affinity was selected and subjected to affinity maturation using a modular engineering approach. Based on the modular architecture of a rebody scaffold, an additional library was constructed by introducing mutations into four variable sites of LRRV5, and rB2 with a binding affinity of 417 nM was selected. Previous studies have revealed that the loops within the cysteine rich domains (CRDs) of hTNF- $\alpha$  receptors are involved in the interaction with hTNF- $\alpha$  based on a structural analysis [23–25]. To further increase the binding affinity, we therefore chose the C-terminal loop region (LRRCT) of rB2 for the library construction, and repeated the same process described



**Fig. 1.** Selection and affinity maturation of repebodies that bind specifically to human TNF- $\alpha$  through a phage display and modular evolution. (A) Variable sites on a repebody used for a library construction. The first library was constructed by randomizing six variable sites on two modules (LRRV2 and LRRV3) (yellow), LRRV5 (orange) and LRRCT (red) were further used for affinity maturation through a modular engineering approach. (B) Amino acid sequences of the selected repebodies against hTNF- $\alpha$  in each round of selection and binding affinity of selected repebodies by ITC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

above. As a result, rB3 with a binding affinity of 49 nM was selected. Amino acid sequences of selected repebodies at each round of affinity maturation are shown in Fig. 1B. Considering the binding affinities of currently used antibodies against hTNF- $\alpha$  ranging from 1 nM to 10 pM [26,27], we chose rB3 for the immunoassays because of its highest binding affinity.

### 3.2. Construction of a mAP-fused repebody

In order to use mAP as a signal generator in an immunoassay, we genetically fused mAP to rB3. Our previous studies revealed that the repebody scaffold is expressed at a high level in bacterial cytoplasm, showing high stability. We fused mAP to the C-terminus of rB3 using a flexible GS linker (Fig. 2A), and expressed the fusion protein in *E. coli* Origami B (DE3) cells. As shown in Fig. 2B, the rB3-mAP fusion protein was confirmed to be well expressed as a soluble monomeric form, displaying a single band with an expected molecular size (90 kDa) through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography. The expression level of the rB3-mAP fusion protein in *E. coli* was estimated to be 12.4 mg L<sup>-1</sup>. It is noteworthy that mAP fused with rB3 showed a higher expression level than free mAP (2.6 mg L<sup>-1</sup>), which implies that rB3 makes a significant contribution to the expression of a genetically fused protein in bacteria.

### 3.3. Catalytic activity and stability of repebody-mAP fusion protein

We checked the catalytic activity and stability of the rB3-mAP fusion protein and free mAP through colorimetric and fluorometric immunoassays using *p*-nitrophenyl phosphate (pNPP) and 4-methylumbelliferyl phosphate (4-MUP) as the substrates, respectively. As shown in Fig. 3A, the signal intensity increased with the increasing concentration of the rB3-mAP fusion protein and free mAP. As expected, a fluorometric immunoassay using 4-

MUP was shown to give rise to approximately ten-times higher signal intensity than a colorimetric immunoassay using pNPP. Interestingly, the rB3-mAP fusion protein displayed almost the same signal intensity as free mAP, indicating that the genetic fusion of rB3 to mAP had a negligible effect on the catalytic activity of the mAP.

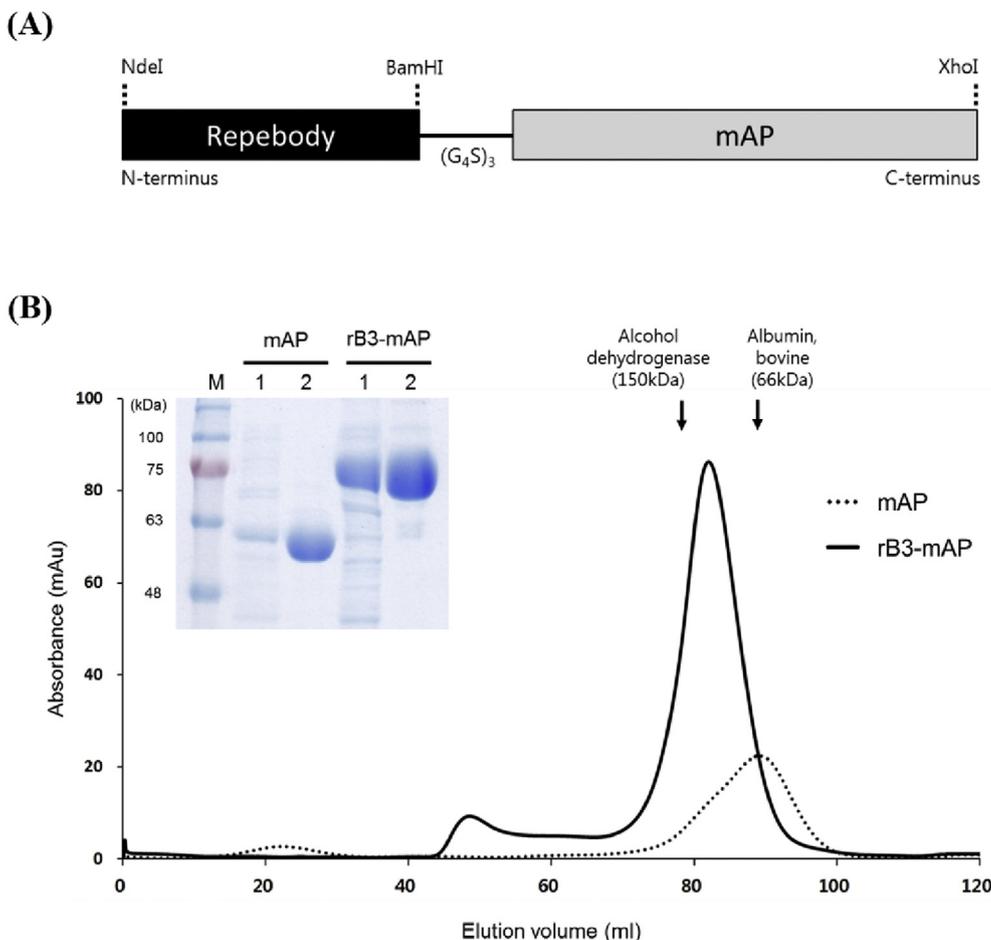
It was reported that free mAP shows thermal instability, resulting in a loss of catalytic activity [11]. We evaluated the long-term storage stability of the rB3-mAP fusion protein. Both free mAP and the rB3-mAP fusion protein were stored at 4 °C without any protective reagents for 25 days and subjected to an assay of the remaining enzyme activity (Fig. 3B). As a result, free mAP and the rB3-mAP showed no significant loss in the catalytic activity even after 25 days. Based on this result, the rB3-mAP fusion protein can be effectively used as an immuno-reagent in an immunoassay with a long-term storage.

### 3.4. Specificity of mAP-fused repebody

Prior to the use for immunoassays, we checked the specificity of the rB3-mAP fusion protein against hTNF- $\alpha$  using a direct immunoassay. hTNF- $\alpha$  was revealed to share an approximately 80% amino acid similarity with mouse TNF- $\alpha$  [28,29]. As a result, mAP-fused rB3 was shown to have a high specificity for hTNF- $\alpha$  without any cross-reactivity for mouse TNF- $\alpha$  or other proteins such as human IL-6 and BSA (Fig. 4). This result indicates that the repebody retained its high specificity for hTNF- $\alpha$  even after genetic fusion of mAP.

### 3.5. Direct immunoassay using repebody-mAP fusion protein

To assess the applicability of an rB3-mAP fusion protein for a direct one-step immunoassay, we first optimized the assay conditions for the highest sensitivity in terms of the concentrations of pNPP and CaCl<sub>2</sub> (Fig. S4A) and the incubation time (Fig. S4B). The



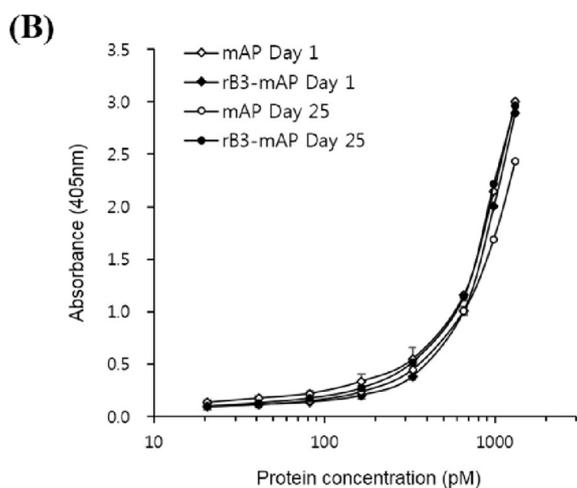
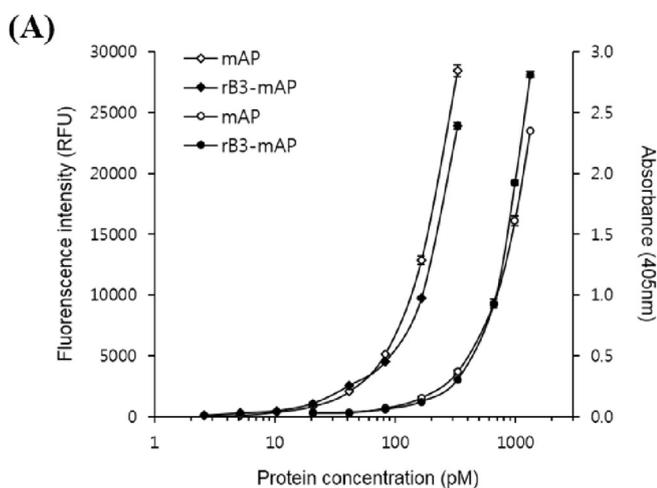
**Fig. 2. Construction and characterization of a monomeric alkaline phosphatase (mAP)-fused reebody.** (A) Schematic of a fusion protein comprising a reebody and monomeric alkaline phosphatase (mAP) using a flexible GS linker. (B) SDS-PAGE (inset) and size exclusion chromatography analyses (superdex 200) of free monomeric alkaline phosphatase (mAP) and rB3-mAP fusion protein produced by *E. coli*. Alcohol dehydrogenase (150 kDa) and bovine albumin (66 kDa) were used as the standard proteins in size exclusion chromatography. M, size marker; 1, non-purified supernatant of protein; 2, purified protein over Ni-NTA column.

optimal incubation time was examined through a direct immunoassay using 1 mM pNPP and 2.5 mM  $\text{CaCl}_2$ . hTNF- $\alpha$  at different concentrations was coated onto a 96-well plate followed by the addition of  $2 \mu\text{g mL}^{-1}$  of rB3-mAP fusion protein. The signal intensity was shown to linearly increase with the increasing concentration of hTNF- $\alpha$  and incubation time. Under optimal conditions, the limit of detection (LOD) for hTNF- $\alpha$  by the rB3-mAP fusion protein was estimated to be  $11.2 \text{ ng mL}^{-1}$  and  $1.2 \text{ ng mL}^{-1}$  for a colorimetric and a fluorometric immunoassay, respectively (Fig. S5). Isothermal titration calorimetry (ITC) experiment revealed that rB3 shows an enthalpy-driven behavior, which appears to be an intrinsic signature for the specific and selective binding mode toward hTNF- $\alpha$  (Fig. S3B). Enthalpy-driven behavior is known to be associated with the formation of noncovalent interactions, such as hydrogen bonds between two interacting molecules. Such hydrogen bonds contribute to the binding affinity, and shape the complementarity between two interacting molecules [30,31]. In this context, rB3 seems to offer a high specificity toward hTNF- $\alpha$  with a high binding affinity. This result also indicates the applicability of the rB3-mAP fusion protein to the analysis of various target proteins through a direct one-step immunoassay.

### 3.6. Sandwich immunoassay using reebody-mAP fusion protein

We tested the utility of the rB3-mAP fusion protein for a sandwich immunoassay. A sandwich immunoassay requires a capture

and detection antibody pair with different epitopes on a target analyte. The concentration of each antibody also influences the sensitivity of a sandwich immunoassay. hTNF- $\alpha$  is a homotrimer and forms a complex with an anti-hTNF- $\alpha$  antibody at a ratio of 2:1 or 3:1 [32]. In contrast, we observed that the binding ratio between rB3 and hTNF- $\alpha$  is 1:1. We therefore expected that an anti-hTNF- $\alpha$  antibody for a sandwich immunoassay would be able to bind to a different site of hTNF- $\alpha$  from the rB3-binding site. To check this possibility, we chose Mab1 as a capture antibody. Mab1 is a monoclonal antibody against hTNF- $\alpha$  and is widely used as a capture antibody in a sandwich immunoassay together with biotinylated Mab11 as a detection antibody [33]. We optimized the assay conditions with respect to the concentrations of the capture antibody and rB3-mAP fusion protein, and observed that  $2 \mu\text{g mL}^{-1}$  of Mab1 and  $2 \mu\text{g mL}^{-1}$  of rB3-mAP fusion protein led to the best assay performance. Under optimized conditions, we evaluated the sensitivity of a sandwich immunoassay using Mab1 and the rB3-mAP fusion protein. Mab1 was coated onto a 96-well plate, followed by the addition of serially diluted hTNF- $\alpha$ , ranging from 0 to  $10 \text{ ng mL}^{-1}$ , and the rB3-mAP fusion protein ( $2 \mu\text{g mL}^{-1}$ ). The signal intensity was then measured using a fluorescent immunoassay (Fig. 5). The signal intensity showed a direct relationship with the concentration of hTNF- $\alpha$  at a log scale, with a good correlation coefficient ( $R^2 = 0.9998$ ). The LOD of the hTNF- $\alpha$  was estimated to be  $216 \text{ pg mL}^{-1}$ , which corresponds to a 5.6-fold higher sensitivity than a direct immunoassay. The dynamic range by rB3-mAP was

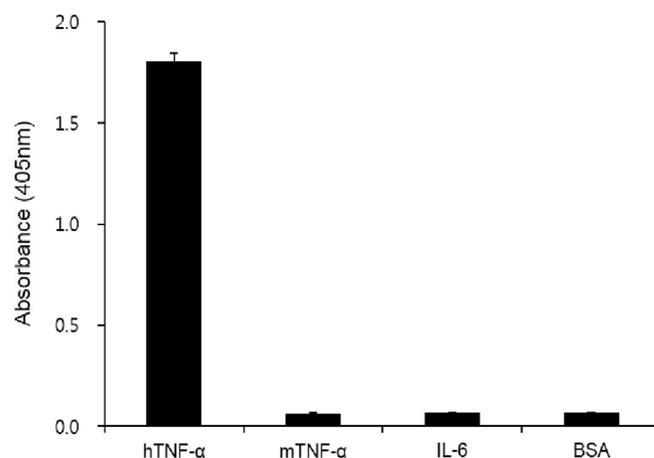


**Fig. 3. Activity and storage stability of a mAP-fused rebody** (A) The enzyme activities of the rB3-mAP fusion protein and free mAP were measured using colorimetric (closed and open circles) and fluorometric (closed and open diamonds) immunoassays, respectively. (B) Storage stability of the rB3-mAP fusion protein and free mAP. The rB3-mAP and free mAP were stored at 4 °C for 25 days, and the remaining activity was measured through a colorimetric assay. The error bars indicate the SD in the triplicate experiments.

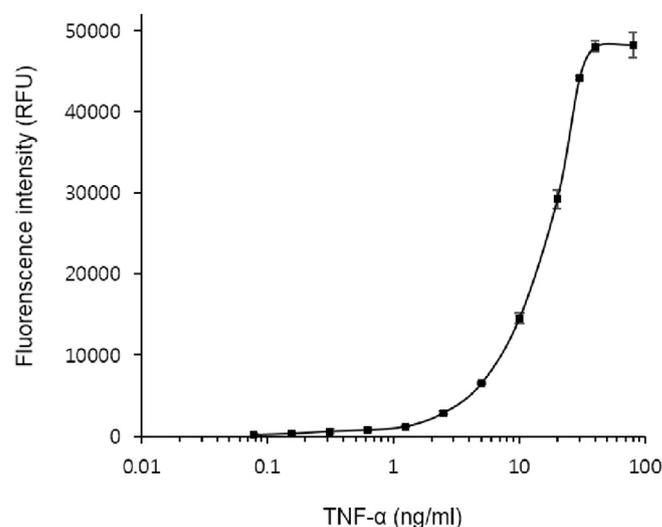
shown to be between 0.6 and 27.2 ng mL<sup>-1</sup>. It is likely that Mab1 and the rB3-mAP fusion protein have different binding sites on hTNF- $\alpha$  with a negligible steric hindrance, resulting in a high sensitivity. The detection limit was relatively low compared to other hTNF- $\alpha$  immunoassay methods. Based on the results, the present approach can be effectively used in a sandwich immunoassay, considering that commercial anti-hTNF- $\alpha$  antibodies were used for detection of hTNF- $\alpha$  in previous studies [34,35].

### 3.7. Performance of mAP as a signal generator

Dimeric *E. coli* alkaline phosphatase (dAP) has been widely used as a signal generator in immunoassays owing to its high catalytic activity and thermal stability [36]. We compared the performance of mAP as a signal generator with commonly used dAP. To do so, we genetically fused rB3 to the N-terminus of a dAP variant with double mutations (D153G and D330 N). The double mutant of the dAP was reported to have much higher catalytic activity than the wild-type dAP [37]. As a result, the rB3-dAP fusion protein was



**Fig. 4. Specificity of rB3-mAP fusion protein.** Specificity of the rB3-mAP fusion protein was measured through a colorimetric immunoassay for hTNF- $\alpha$ , mouse TNF- $\alpha$  (mTNF- $\alpha$ ), interleukin-6 (IL-6), and bovine serum albumin (BSA). The error bars indicate the SD in the triplicate experiments.



**Fig. 5. Detection sensitivity of rB3-mAP fusion protein for hTNF- $\alpha$ .** Sandwich immunoassay using the rB3-mAP fusion protein and Mab1 as a capture antibody. The signal intensity was measured using a fluorometric immunoassay. The error bars indicate the SD in the triplicate experiments.

shown to be well-expressed as a soluble form in *E. coli* as an rB3-mAP fusion protein, exhibiting an expected molecular mass through SDS-PAGE. Size exclusion chromatography confirms that the rB3-dAP fusion protein has a dimeric structure (Fig. S6). The expression levels of the rB3-dAP fusion protein and free dAP were estimated to be 46 and 28 mg L<sup>-1</sup>, respectively, supporting a significant contribution of rB3 to an increase in the expression level of the fusion protein. The expression level of rB3-dAP fusion protein was shown to be much higher than those of scFv-dAP and nanobody-dAP fusion proteins. To evaluate the sensitivity of the rB3-dAP fusion protein in a sandwich immunoassay, serially diluted hTNF- $\alpha$  was coated onto a 96-well plate, followed by the addition of the rB3-dAP fusion protein and substrate, and the signal intensity was then measured. Considering a high catalytic activity and avidity of dAP, the rB3-dAP fusion protein was expected to lead to a higher sensitivity than the rB3-mAP fusion protein. However, the rB3-dAP fusion protein exhibited a relatively low sensitivity for hTNF- $\alpha$  in a sandwich immunoassay compared to the rB3-mAP fusion protein, resulting in an LOD of 823 pg mL<sup>-1</sup> for hTNF- $\alpha$ ,

**Table 1**  
Immunoassay of hTNF- $\alpha$  spiked in a medium supplemented with 10% FBS using an rB3-mAP fusion protein.

Spiked TNF- $\alpha$ (ng/ml)	Mean $\pm$ SD (ng/ml)	Recovery (%)	CV (%)
0.63	0.67 $\pm$ 0.03	101	4.2
1.25	1.35 $\pm$ 0.13	106	9.8
2.5	2.43 $\pm$ 0.17	101	5.9
5	4.90 $\pm$ 0.09	98	4.5

which corresponds to a 3.8-fold lower sensitivity than the rB3-mAP fusion protein (Fig. S7). Furthermore, the rB3-dAP in a direct immunoassay was also shown to have lower sensitivity than the rB3-mAP (Fig. S8 and Table S1). It seems that a dimeric form of the rB3-dAP fusion protein caused a steric hindrance to the binding of rB3 to hTNF- $\alpha$ , consequently resulting in a reduced sensitivity in the direct and sandwich immunoassays. As an approach to avoiding a steric hindrance, a monomeric form of *E. coli* alkaline phosphatase was constructed using amino acid substitutions, however, the catalytic activity and stability of the resulting enzyme were significantly reduced when compared with the wild-type [38]. Therefore, mAP seems to be more suitable as a signal generator than dimeric AP in a sandwich immunoassay.

### 3.8. Validation test for rebody-mAP fusion protein

To check the practical utility of the rB3-mAP fusion protein in an immunoassay, various concentrations of hTNF- $\alpha$  were spiked in a cell culture medium supplement with 10% FBS, and its concentration was determined through a sandwich immunoassay using the rB3-mAP fusion protein. It was known that proteins in a serum interfere with the assay of hTNF- $\alpha$ , resulting in a decreased sensitivity [39]. We used 10 ng mL<sup>-1</sup> of hTNF- $\alpha$  as a control, and determined the sensitivity based on the signal difference between 10 ng mL<sup>-1</sup> hTNF- $\alpha$  in a blocking solution and 10 ng mL<sup>-1</sup> hTNF- $\alpha$  in RPMI-10% FBS (Fig. S9). As shown in Table 1, the measured concentrations of hTNF- $\alpha$  in RPMI-10% FBS were well correlated with the spiked concentrations, exhibiting high average recoveries (98–106%) and a coefficient of variation (CV) ranging from 4.2 to 9.8%. The results indicate that it is likely that the rB3-mAP fusion protein can detect a hTNF- $\alpha$  level of as low as 630 pg mL<sup>-1</sup> in a complex solution. In addition, the hTNF- $\alpha$  concentration can be determined based on a standard curve with a high level of sensitivity and precision through a sandwich immunoassay.

## 4. Conclusions

We demonstrated that a monomeric alkaline phosphatase (mAP)-fused rebody can be effectively used in an immunoassay with high sensitivity. The rB3-mAP fusion protein was expressed at high level in *E. coli*, and retained a binding affinity of rB3 and catalytic activity of mAP, implying a negligible effect of genetic fusion on the properties of the rB3 and mAP. The rB3-mAP fusion protein allowed for a sensitive detection of hTNF- $\alpha$  in a sandwich immunoassay, which indicates the broad utility and potential of a mAP-fused rebody as a cost-effective immuno-reagent in an immunoassay based on easy production through bacterial expression system and high storage stability. Based on the results, a monomeric AP is likely to be more suitable as a signal generator than a dimeric AP in an immunoassay. Selection and affinity maturation of a target-specific rebody can be easily carried out through a phage display and modular engineering approach. It is therefore anticipated that a mAP-fused rebody can be widely used as a new format of immuno-reagent for an immunoassay.

## Acknowledgements

This research was supported by the Midcareer Researcher Program (NRF-2014R1A2A1A01004198) and Global Research Laboratory (NRF2015K1A1A2033346) of the National Research Foundation (NRF) funded by the Ministry of Science, ICT and Future Planning, and Brain Korea 21 funded by the Ministry of Education. We would like to thank Tae H. Lee (Yonsei University, Korea) for providing the hTNF- $\alpha$  gene.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.aca.2016.11.013>.

## References

- [1] H. Oyama, E. Tanaka, T. Kawanaka, I. Morita, T. Niwa, N. Kobayashi, Anti-idiotypic scFv-enzyme fusion proteins: a clonable analyte-mimicking probe for standardized immunoassays targeting small biomarkers, *Anal. Chem.* 85 (2013) 11553–11559.
- [2] A. Jeanson, J.M. Cloes, M. Bouchet, B. Rentier, Comparison of conjugation procedures for the preparation of monoclonal antibody-enzyme conjugates, *J. Immunol. Methods* 111 (1988) 261–270.
- [3] F.W. Krainer, A. Glieder, An updated view on horseradish peroxidases: recombinant production and biotechnological applications, *Appl. Microbiol. Biotechnol.* 99 (2015) 1611–1625.
- [4] J.L. Liu, D. Zabetakis, A.B. Lee, E.R. Goldman, G.P. Anderson, Single domain antibody-alkaline phosphatase fusion proteins for antigen detection - analysis of affinity and thermal stability of single domain antibody, *J. Immunol. Methods* 393 (2013) 1–7.
- [5] M. Shu, Y. Xu, X. Liu, Y. Li, Q. He, Z. Tu, J. Fu, S.J. Gee, B.D. Hammock, Anti-idiotypic nanobody-alkaline phosphatase fusion proteins: development of a one-step competitive enzyme immunoassay for fumonisin B1 detection in cereal, *Anal. Chim. Acta* 924 (2016) 53–59.
- [6] M.D. Swain, G.P. Anderson, J. Serrano-González, J.L. Liu, D. Zabetakis, E.R. Goldman, Immunodiagnostic reagents using llama single domain antibody-alkaline phosphatase fusion proteins, *Anal. Biochem.* 417 (2011) 188–194.
- [7] S.H. Wang, J. Bin Zhang, Z.P. Zhang, Y.F. Zhou, R.F. Yang, J. Chen, Y.C. Guo, F. You, X.E. Zhang, Construction of single chain variable fragment (scFv) and BiscFv- alkaline phosphatase fusion protein for detection of *Bacillus Anthracis*, *Anal. Chim. Acta* 78 (2006) 997–1004.
- [8] G.L. Kumari, R.N. Dhir, Comparative studies with penicillinase, horseradish peroxidase, and alkaline phosphatase as enzyme labels in developing enzyme immunoassay of cortisol, *J. Immunoass. Immunochem.* 24 (2003) 173–190.
- [9] M. Iijima, M. Yamamoto, N. Yoshimoto, T. Niimi, S. Kuroda, Bio-nanocapsules for signal enhancement of alkaline phosphatase-linked immunosorbent assays, *Biosci. Biotechnol. Biochem.* 77 (2013) 843–846.
- [10] N.E. Coe Clough, P.J. Hauer, Using polyclonal and monoclonal antibodies in regulatory testing of biological products, *ILAR J.* 46 (2005) 300–306.
- [11] D.H. Lee, S.L. Choi, E. Rha, S.J. Kim, S.J. Yeom, J.H. Moon, S.G. Lee, A novel psychrophilic alkaline phosphatase from the metagenome of tidal flat sediments, *BMC Biotechnol.* 15 (2015) 1.
- [12] W. Heu, J.M. Choi, J.J. Lee, S. Jeong, H.S. Kim, Protein binder for affinity purification of human immunoglobulin antibodies, *Anal. Chem.* 86 (2014) 6019–6025.
- [13] D.E. Hwang, J.H. Ryou, J.R. Oh, J.W. Han, T.K. Park, H.S. Kim, Anti-human VEGF rebody effectively suppresses choroidal neovascularization and vascular leakage, *PLoS One* 11 (2016) e0152522.
- [14] H.Y. Kim, J. Lee, N. Kim, W.D. Heo, H.S. Kim, Tracking protein-protein interaction and localization in living cells using a high-affinity molecular binder, *Biochem. Biophys. Res. Commun.* 470 (2016) 857–863.
- [15] J.J. Lee, H.J. Choi, M. Yun, Y. Kang, J.E. Jung, Y. Ryu, T.Y. Kim, Y.J. Cha, H.S. Cho, J.J. Min, C.W. Chung, H.S. Kim, Enzymatic prenylation and oxime ligation for the synthesis of stable and homogeneous protein-drug conjugates for targeted therapy, *Angew. Chem. Int. Ed.* 54 (2015) 12020–12024.
- [16] S.C. Lee, K. Park, J. Han, J.J. Lee, H.J. Kim, S. Hong, W. Heu, Y.J. Kim, J.S. Ha, S.G. Lee, H.K. Cheong, Y.H. Jeon, D. Kim, H.S. Kim, Design of a binding scaffold based on variable lymphocyte receptors of jawless vertebrates by module engineering, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 3299–3304.
- [17] J.J. Lee, H.J. Kim, C.S. Yang, H.H. Kyeong, J.M. Choi, D.E. Hwang, J.M. Yuk, K. Park, Y.J. Kim, S.G. Lee, D. Kim, E.K. Jo, H.K. Cheong, H.S. Kim, A high-affinity protein binder that blocks the IL-6/STAT3 signaling pathway effectively suppresses non-small cell lung cancer, *Mol. Ther.* 22 (2014) 1254–1265.
- [18] S. Laing, A. Hernandez-Santana, J. Sasmannshausen, D. Asquith, I. McInnes, K. Faulds, D. Graham, Quantitative detection of human tumor necrosis factor alpha by a resonance raman enzyme-linked immunosorbent assay, *Anal. Chem.* 83 (2011) 297–302.
- [19] C. Petrovas, S.M. Daskas, E.S. Lianidou, Determination of tumor necrosis

- factor- $\alpha$  (TNF- $\alpha$ ) in serum by a highly sensitive enzyme amplified lanthanide luminescence immunoassay, *Clin. Biochem.* 32 (1999) 241–247.
- [20] A. Paguio, P. Stecha, K.V. Wood, F. Fan, Improved dual-luciferase reporter assays for nuclear receptors, *Curr. Chem. Genomics.* 4 (2010) 43–49.
- [21] C. Lai, X. Jiang, X. Li, Development of luciferase reporter-based cell assays, *Assay. Drug Dev. Technol.* 4 (2006) 307–315.
- [22] B. Goffe, J.C. Cather, Etanercept: an overview, *J. Am. Acad. Dermatol.* 49 (2003) 105–111.
- [23] J.L. Bodmer, P. Schneider, J. Tschopp, The molecular architecture of the TNF superfamily, *Trends biochem. Sci.* 27 (2002) 19–26.
- [24] M. Branschädel, A. Aird, A. Zappe, C. Tietz, A. Krippner-Heidenreich, P. Scheurich, Dual function of cysteine rich domain (CRD) 1 of TNF receptor type 1: conformational stabilization of CRD2 and control of receptor responsiveness, *Cell. Signal* 22 (2010) 404–414.
- [25] J.H. Naismith, T.Q. Devine, B.J. Brandhuber, S.R. Sprang, Crystallographic evidence for dimerization of unliganded tumor necrosis factor receptor, *J. Biol. Chem.* 270 (1995) 13303–13307.
- [26] D.J. Shealy, A. Cai, K. Staquet, A. Baker, E.R. Lacy, L. Johns, O. Vafa, G. Gunn 3rd, S. Tam, S. Sague, D. Wang, M. Brigham-Burke, P. Dalmonte, E. Emmell, B. Pikounis, P.J. Bugelski, H. Zhou, B.J. Scallon, J. Giles-Komar, Characterization of golimumab, a human monoclonal antibody specific for human tumor necrosis factor alpha, *MAbs* 2 (2010) 428–439.
- [27] H. Szmajcinski, Z. Murtaza, J.R. Lakowicz, Time-resolved fluorometric method for one-step immunoassays using plasmonic nanostructures, *J. Phys. Chem. C* 114 (2010) 7236–7241.
- [28] D. Caput, B. Beutler, K. Hartog, R. Thayer, S. Brown-Shimer, A. Cerami, Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators, *Proc. Natl. Acad. Sci. U. S. A.* 83 (1986) 1670–1674.
- [29] A. Jonsson, H. Wällberg, N. Herne, S. Ståhl, F.Y. Frejd, Generation of tumour-necrosis-factor-alpha-specific affibody molecules capable of blocking receptor binding in vitro, *Biotechnol. Appl. Biochem.* 54 (2009) 93–103.
- [30] S. Núñez, J. Venhorst, C.G. Kruse, Target-drug interactions: first principles and their application to drug discovery, *Drug Discov. Today* 17 (2012) 10–22.
- [31] E. Freire, Isothermal titration calorimetry: controlling binding forces in lead optimization, *Drug Discov. Today Technol.* 1 (2004) 295–299.
- [32] T. Kohno, L.T.T. Tam, S.R. Stevens, J.S. Louie, Binding characteristics of tumor necrosis factor receptor-Fc fusion proteins vs anti-tumor necrosis factor mAbs, *J. Investig. Dermatol. Symp. Proc.* 12 (2007) 5–8.
- [33] R. Rieger, D. Whitacre, M.J. Cantwell, C. Prussak, T.J. Kipps, Chimeric form of tumor necrosis factor-alpha has enhanced surface expression and antitumor activity, *Cancer Gene Ther.* 16 (2009) 53–64.
- [34] N. Theilacker, E.E. Roller, K.D. Barbee, M. Franzreb, X. Huang, Multiplexed protein analysis using encoded antibody-conjugated microbeads, *J. R. Soc. Interface* 8 (2011) 1104–1113.
- [35] J.L. Miller, K. Velmurugan, M.J. Cowan, V. Briken, The type I NADH dehydrogenase of *Mycobacterium tuberculosis* counters phagosomal NOX2 activity to inhibit TNF-alpha-mediated host cell apoptosis, *PLoS Pathog.* 6 (2010) e1000864.
- [36] M.H. Le Du, C. Lamoure, B.H. Muller, O.V. Bulgakov, E. Lajeunesse, A. Ménez, J.-C. Boulain, Artificial evolution of an enzyme active site: structural studies of three highly active mutants of *Escherichia coli* alkaline phosphatase, *J. Mol. Biol.* 316 (2002) 941–953.
- [37] B.H. Muller, C. Lamoure, M.H. Le Du, L. Cattolico, E. Lajeunesse, F. Lemaître, A. Pearson, F. Ducancel, A. Ménez, J.C. Boulain, Improving *Escherichia coli* alkaline phosphatase efficacy by additional mutations inside and outside the catalytic pocket, *Chembiochem* 2 (2001) 517–523.
- [38] R.R. Boulanger, E.R. Kantrowitz, Characterization of a monomeric *Escherichia coli* alkaline phosphatase formed upon a single amino acid substitution, *J. Biol. Chem.* 278 (2003) 23497–23501.
- [39] M. Herrmann, T. Veres, M. Tabrizian, Quantification of low-picomolar concentrations of TNF- $\alpha$  in serum using the dual-network microfluidic ELISA platform, *Anal. Chem.* 80 (2008) 5160–5167.