Supplementary information for

Nanobody as solubilization chaperone for the expression and purification of inclusion-body prone proteins

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1. Materials and experimental methods

1.1 Chemicals and reagents

Yeast powder, peptone, isopropyl thiogalactoside (IPTG), ampicillin (Amp), kanamycin (Kan), recombinant human IL-17A and IL-6, bicinchoninic acid (BCA) protein assay kit, and enzyme-linked immunosorbent assay reagents (ELISA) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China); PrimeSTAR Max premix® DNA polymerase and restriction enzymes were purchased from TaKaRa (Beijing, China). Plasmid mini kit I, cycle pure kit, and gel extraction kit were purchased from Omega Bio-Tek (Guangzhou, China); Ni Sepharose™ 6 Fast Flow affinity chromatographic media was purchased from GE Healthcare (MA, USA). The human skin fibroblasts (HSF) were purchased from iCell Bioscience Inc. (Shanghai, China). High-glucose Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Thermo Scientific (Waltham, MA). Cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Beijing, China). Tetrapeptide of EPEA was synthesized by Ontores Biotechnologies Co., Ltd (Hangzhou, China). All other chemicals were of analytical grade and used without further purification.

1.2 Amino acid sequences of recombinant proteins

IL-17A-Nb

AQLQLVESGGGLVQAGGSLRLSCAASERTISNYDMGWFRQAPGKERELIAAD
ISWSALNTNYADSVKGRFTISRDNAKNMVYLQMNNLKPEDTAVYYCAARRS
GYASFDNWGQGTLVTVSS

IL-6-Nb

AQVQLVESAGGLVQPGGSLRLSCAASGIIFSINAMGWYRQAPGKRRELVADI
MPYGSTEYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCHSYDPRG
DDYWGQGTQVTVSSEPKTPKPQPQPQPQPQPDPTTEHHHHHH

Syn2-Nb

QGQLVESGGGSVQAGGSLRLSCAASGIDSSSYCMGWFRQRPGKEREGVARIN
GLGGVKTAYADSVKDRFTISRDNAENTVYLQMNSLKPEDTAIYYCAAKFSPG
YCGGSWSNFGYWGQGTQVTVSSGGGGSHHHHHHHH

IL-17A

GITIPRNPGCPNSEDKNFPRTVMVNLNIHNRNTNTNPKRSSDYYNRSTSPWNL
HRNEDPERYPSVIWEAKCRHLGCINADGNVDYHMNSVPIQQEILVLRREPPH
CPNSFRLEKILVSVGCTCVTPIVHHVAGGGGSHHHHHHHH

IL-6

PVPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCESSK
EALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQNRFES
SEEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLLTKLQAQNQWLQ
DMTTHLILRSFKEFLQSSLRALRQM

IL-6-EPEA

PVPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCESSK
EALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQNRFES
SEEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLLTKLQAQNQWLQ
DMTTHLILRSFKEFLQSSLRALRQMEPEA

IL-17A-EPEA

GITIPRNPGCPNSEDKNFPRTVMVNLNIHNRNTNTNPKRSSDYYNRSTSPWNL
HRNEDPERYPSVIWEAKCRHLGCINADGNVDYHMNSVPIQQEILVLRREPPH
CPNSFRLEKILVSVGCTCVTPIVHHVAEPEA

*Sequences with black font are the original amino acid sequence of the recombinant proteins. Sequences highlighted in green encode a His-tag or an EPEA-tag.

1.2 Plasmids and strains

E. coli Shuffle T7 Express competent cells were purchased from New England BioLabs Inc. (Ipswich, UK). *E. coli* DH5α was obtained from TaKaRa (Beijing, China). Plasmids pET-28a and pET-21a are commercially available from Novagen (Germany).

1.3 Construction of prokaryotic expression vectors

Genes of anti-IL-17A Nb (termed IL-17A-Nb) and anti-IL-6 Nb (termed IL-6-Nb) were separately cloned into the pET-21a plasmid using the NdeI and XhoI restriction sites. Genes of IL-17A (termed IL-17A), IL-6 (termed IL-6), and Nb towards EPEA-tag (termed Syn2-Nb) were cloned into the pET-28a plasmid using the NcoI and XhoI restriction sites, respectively. All the above plasmids were constructed by Sangon Biotech Ltd. (Shanghai, China).

With the following primers:

F-5'-GATACATATGGGGATCACCATCCCGCGTAACCCAGGTTG-3', R-5'-GACACTCGAGTTATTATGCTTCCGGTTCCGCAACGTGGTGAAC-3', the polymerase chain reaction (PCR) amplification was performed by using the plasmid pET-21a-IL-17A as a template. After being purified by cycle pure kit, the PCR amplicons were digested with NdeI and XhoI restriction endonucleases, then, the DNA fragments were inserted into pET-21a digested with the same enzymes to produce the C-terminal EPEA-tagged IL-17A recombinant plasmid (termed IL-17A-EPEA). Likewise, the gene encoding C-terminal EPEA-tagged IL-6 (termed IL-6-EPEA) was cloned by PCR amplification with the primers: F-5'-AGATATACATATGGCACCGGTTCCGC-3', and

R-5'-TTGGTGCTCGAGATTACGCTTCCGGTTCCATTTGACGCAGTG-3', the enzyme-digested products were inserted into the pET-21a by using the same restriction sites as above. The constructed plasmids were sequenced by Sangon Biotech Ltd. (Shanghai, China).

1.4 Methods of co-expression and purification

1.4.1 Co-expression experiments with corresponding antigen-cognate Nbs

Two sequential transformation steps were executed to obtain the strain that co-expresses the IL and its Nb. Briefly, in the first step, IL-17A-Nb plasmid (pET-21a) was transformed into the *E. coli* Shuffle T7 competent cells and positive colonies were

selected on selective plates containing ampicillin (Amp). Competent cells were then generated from these transformants by the standard calcium chloride method. These IL-17A-Nb plasmid containing competent cells were then used for a second transformation with IL-17A plasmid (pET-28a). Colonies were selected using double selection makers, Amp and kanamycin (Kan). The same protocol was followed to generate the IL-6 & IL-6-Nb co-expression system.

Positive clones (IL-6 & IL-6-Nb or IL-17A & IL-17A-Nb) were incubated into LB liquid medium containing 100 μg/mL Amp and 15 μg/mL Kan at 180 rpm and 37 °C until the optical density of OD_{600} = 0.6-0.8. Then 2 mL of the culture was transferred into 200 mL fresh TB liquid medium for cultivation until the OD_{600} reached 5-6. After that, the IPTG was added to a final concentration of 0.25 mM, followed by shaking at 180 rpm and 17 °C for 20 h. The bacteria were harvested by centrifugation at 9000 ×g for 5 min. The cells were then re-suspended in phosphate buffer (PBS containing 20 mM imidazole, pH 7.4) at 4 °C and lysed by the high-pressure homogenization (700 MPa). After centrifugation at 10000 ×g for 30 min, the supernatant was purified by Ni-immobilized metal ion affinity chromatography (Ni-IMAC). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and LTQ Orbitrap liquid chromatography-tandem mass spectrometry (LC-MS, Thermo Scientific) were used to characterize the purified proteins.

1.4.2 Syn2-Nb as a versatile chaperone to co-express with EPEA-tagged antigens

To further explore the application potential of Nb as a “molecular chaperone”, another two co-expression systems (IL-17A-EPEA & Syn2-Nb or IL-6-EPEA & Syn2-Nb) were constructed following the protocol specified in 1.4.1. The difference with the previous method is that the IL contained the EPEA-tag at its C terminal end, which is recognized specifically by the Syn2-Nb as the co-expressed chaperone in this system.

The co-expression methods were similar to those explained in section 1.4.1, except that the final concentration of IPTG was adjusted to 0.1 mM. Following the cell harvest, cell lysis, and centrifugation, the crude bacterial lysate extractions were captured via

Ni-IMAC. After loading, the adsorbent was amply washed with PBS (containing 40 mM imidazole, pH 7.4) until protein could no longer be detected in the effluent. We then proceeded with the on-column competitive elution to separate the target antigens from the protein complexes. Briefly, 10 column volumes of a 60 mM EPEA peptide solution (in PBS, pH 7.4) were passed through the column at 2 mL/min for 5 cycles. The circulating solution was then collected to obtain the competitively eluted proteins by ultrafiltration (Amicon Ultra-15, MWCO 3 kDa, Millipore, USA). Notably, the oligopeptides dissolved in the filtrate could be reused after concentrating by lyophilization.

1.5 Characterization of EPEA-tagged proteins

1.5.1 Experimental method of LC-MS

The HPLC system consisted of a degasser and a quaternary surveyor MS pump (Thermo Scientific) was connected to the LTQ Orbitrap mass spectrometry (Thermo Scientific) and used for LC-MS analysis. Briefly, 10 μL of protein samples (1 mg/mL) was injected and separated by a Hypersil GOLD™ C18 liquid chromatography column (Thermo Scientific). 0.1% FA in water and acetonitrile were used as mobile phases A and B, respectively. A linear gradient was generated from 20% to 80% mobile phase B in 30 min at a column temperature of 25 °C, followed by electron spray ionization mass spectrometry (ESI-MS). The LTQ-Orbitrap mass was operated in survey scan mode. Full mass scan performed in the Orbitrap analyzer was acquired from m/z 200 to 2000 (Resolution = 60000 at m/z 400). Xcalibur and BioPharma Finder Mass Informatics Platform (Thermo Scientific) were used for spectrum deconvolutions for protein characterization. Default SW Xtract (sliding windows and isotopically resolved) processing method was used to identify the intact protein, with m/z range of 200~2000 and merge tolerance of 30 ppm.

1.5.2 Native-PAGE and mass spectrometric analysis

Interactions between recombinant EPEA-tagged IL and its cognate Nb (i.e., IL-17A-EPEA vs. IL-17A-Nb and IL-6-EPEA vs. IL-6-Nb) were analyzed by native-PAGE

(12% (w/v) Tris-glycine separation gels). Nbs specific for IL-6 or IL-17A were produced as described previously.[1] EPEA-tagged ILs (1 mg/mL in 20 mM MES buffer, pH 7.4) were mixed with their cognate Nbs (2 mg/mL in PBS, pH 7.4) at preset ratios. Then, 5× native sample buffer (100 mM Tris-HCl, 25% glycerol, 0.1% Bromo Phenol Blue, pH 6.8) was added to the mixed samples, and 10 μL aliquots were loaded on the gel. Electrophoresis was performed in an ice bath at 120 V for 4 h. Besides, LC-MS was used to identify the molecular mass of both the soluble IL-17A-EPEA and IL-6-EPEA.

1.5.3 Surface plasmon resonance (SPR) experiments

SPR experiments were performed on a Biacore T200 (GE Healthcare, USA). IL-6-Nbs or IL-17A-Nbs were immobilized on the CM5 sensor chip via EDC/NHS chemistry. The running buffer for all experiments was 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and 0.005% (v/v) Nonidet P20. The analytes were diluted in running buffer to the respective starting concentrations and loaded for 120 s over the Nb-immobilized surface with dissociation times of 300 s. The surface was regenerated using a pulse of 50 mM Gly-HCl buffer (pH 1.5) after each injection. Data analysis was performed by using the Biacore T200 Evaluation Software (version 1.0). All data were fit to a simple 1:1 binding model.

1.5.4 Circular dichroism (CD) spectroscopy

CD measurement was carried out at room temperature on the MOS-500 spectrometer (Bio-Logic, France) for EPEA-tagged proteins (0.5 mg/mL in 20 mM MES). Far-UV spectra (from 180 to 260 nm) were acquired in a 1-mm-path-length cell with a step size of 1 nm. Three scanning acquisitions were collected and averaged to yield each CD spectrum. Buffer spectra were subtracted from all sample spectra before plotting.

1.5.5 Enzyme-linked immune sorbent assay (ELISA)

Sandwich ELISA was performed to determine the antigenicity of IL-17A-EPEA and IL-6-EPEA. In brief, the amounts of the purified ILs were firstly determined with BCA. IL samples were then diluted to serial concentrations (25, 50, 100, 150, 200 pg/mL)

according to the BCA concentration measurement. Thereafter, the IL amount in each sample was quantified again by ELISA kit and compared to the predetermined concentrations by BCA. The refolded IL-17A and IL-6 were used as control, the refolding of ILs referred to the methods reported in the previous literatures.[2, 3]

1.5.6 Biological activity assay

The biological activity of the recombinant IL-17A-EPEA was measured by a stimulus-secretion of IL-6 on HSF. Briefly, HSF cells were seeded at 5000 cells/well in a 96-well plate in 100 μL culture medium and allowed to grow for 12 h with 5% CO_2. Then the medium supernatant was replaced by fresh medium spiked with IL-17A-EPEA at various concentrations (0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 50, 100, and 200 ng/mL). Following another 24 h incubation, the secreted IL-6 in the supernatant medium was quantified by ELISA.

In vitro A431 cell (lab cell) proliferation assay for biological activity measurement of IL-6-EPEA was conducted. Briefly, the cells were seeded in a 96-well plate in 100 μL medium at 5000 cells/well for 12 h in a 5% CO_2 humidified incubator. Then the supernatant was replaced by fresh DMEM spiked with different concentrations of IL-6-EPEA (1, 5, 10, 20, 50, 100, 200, and 300 ng/mL). Cell proliferation was analyzed by CCK-8 kit following a 48 h culture. Three sets of parallel replicates were set up in all groups, whereas commercial IL-17A and IL-6 were used as control.

2. Results and discussions

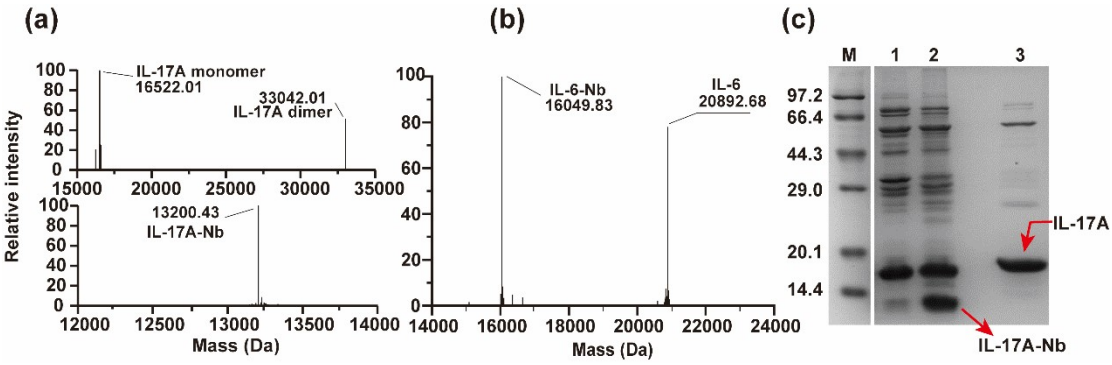

Fig. S1 The deconvoluted mass spectra from LC–MS analysis of the solubilized complexes of (a) IL-17A & IL-17A-Nb and (b) IL-6 & IL-6-Nb. (c) On-column elution of IL-17A from the IL-17A & IL-17A-Nb complex with Gly-HCl (pH 1.5). M: marker; 1: protein complex loaded IMAC gel sample washed by Gly-HCl (pH 1.5); 2: initial IMAC gel sample loaded with protein complex; 3: 500 mM imidazole eluted protein from the IMAC gel that has already been washed by Gly-HCl.

The deconvoluted mass spectrum (Figs. S1a and S1b) confirmed that the molecular masses of each component derived from the purified IL & cognateNb complexes are in good agreement with the theoretical masses calculated from their primary sequences (16522.05 Da for monomer IL-17A, 20892.67 Da for IL-6). Notably, two peaks appeared in the IL-17A mass spectrum, suggesting that two forms of the recombinant IL-17A existed in the sample: the monomer and the interchain disulfide-bonded linked homodimer. Very similar results have been reported in earlier work on recombinant IL-17A expressed either in *E. coli or* in the mammalian cell.[2, 4, 5]

The successful on-column disassociation of soluble IL-17A from the IL-17A & IL-17A-Nb occasionally occurred when the elution condition harsh is harsh enough (pH 1.5 of Gly-HCl, Fig. S1c). Because the Ni-NTA Sepharose gel can not be subjected to such stringent elution conditions, in most cases, the eluates are usually the mixture of cognate Nbs and antigens. This phenomenon is particularly evident when the on-column dissociation of IL-6 was conducted (data not shown).

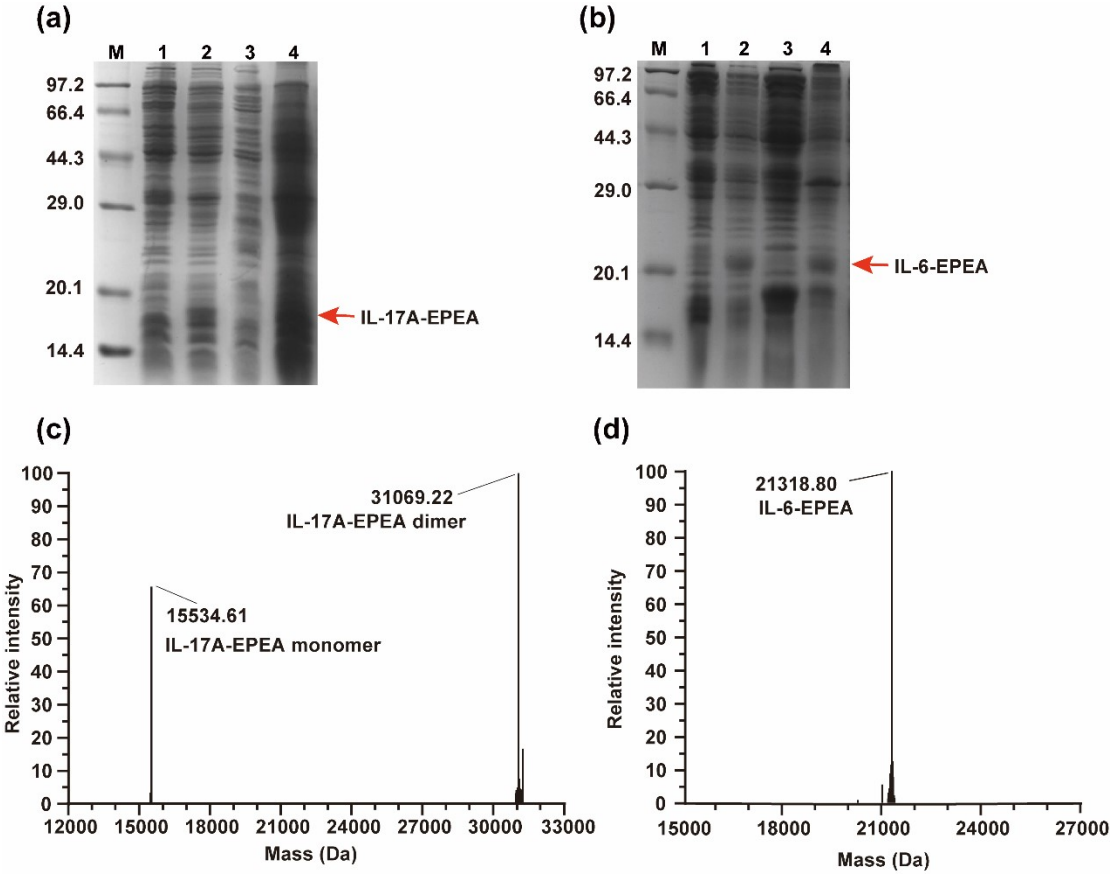

Fig. S2 SDS-PAGE analysis of sole expression of (a) IL-17A-EPEA and (b) IL-6-EPEA in *E. coli*. M: marker; lanes 1 and 2: uninduced and IPTG-induced whole-cell lysate from *E. coli* Shuffle T7 harboring the IL-17A-EPEA and IL-6-EPEA encoded plasmid, respectively; lane 3 and lane 4: supernatant and precipitate of the disrupted cells in lane 2; The deconvoluted mass spectra from LC−MS analysis of the soluble IL-17A-EPEA (c) and IL-6-EPEA (d). The masses of 15534.58, 31069.22, and 21318.80 Da for monomer IL-17A-EPEA, homodimer IL-17A-EPEA, and monomer IL-6-EPEA, respectively, are in good agreement with their theoretical monoisotopic masses of 15534.59, 31069.28, and 21318.84 Da.

The deconvoluted MS for IL-17A-EPEA (Fig. S2c) showed two peaks at 15534.61 Da and 31069.22 Da that matched the theoretical masses of monomer and homodimer, respectively. In addition, the obtained mass of IL-6-EPEA (Fig. S2d) also corresponded to its exact monoisotopic mass. Unlike the two peaks of IL-17A-EPEA observed above, only a monomeric form for IL-6 was observed. Considering that the IL-17A and IL-6 have been confirmed to exist as a homodimer and a monomeric protein in humans, respectively,[6, 7] the IL-6-EPEA prepared here matched precisely the published results. As for the IL-17A-EPEA that existed as a mixture of monomer and intermolecular disulfide-linked homodimer in our study, very similar results have been reported in

earlier work on recombinant IL-17A expressed either in *E. coli* or in mammalian cell.[2, 4, 5] However, heterogenous oligomeric forms seem to be ubiquitous in the expression of cysteine-rich proteins.[8, 9] Thus, additional separation steps may be required to obtain homogeneous populations of monomeric or homodimeric IL-17A proteins when utilizing this co-expression strategy in *E. coli*.

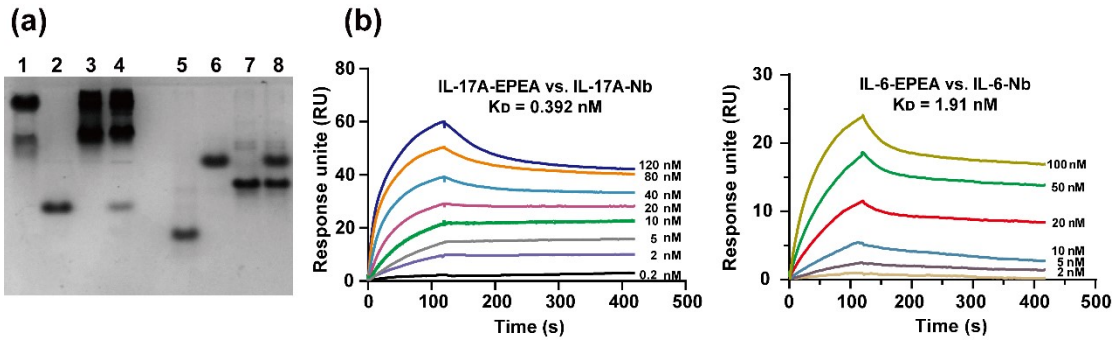

Fig. S3. (a) Native-PAGE analysis of the binding between the EPEA-tagged ILs and their cognate Nbs. Lane 1 and 5: IL-17A-EPEA and IL-6-EPEA; lanes 2 and 6: IL-17A-Nb and IL-6-Nb; lanes 3 and 7: IL-17A-EPEA and IL-17A-Nb mixed in equimolar amounts, IL-6-EPEA and IL-6-Nb mixed in equimolar amounts; lanes 4 and 8: mixed samples with excess Nbs in the same order as those of lanes 3 and 7. (b) Representative SPR sensorgrams displaying the binding of IL-EPEA to its cognate Nbs. Sensorgrams were obtained by the multi-cycle kinetics method.

Notes and references

1. G. Yao, C. Huang, F. Ji, J. Ren, B. Zang and L. Jia, *J. Chromatogr. A*, 2021, **1654**, 462478.
2. B. Wu, J. F. Nemeth, D. J. Janecki, B. Jones, G. Obmolova, T. J. Malia, A. Baker, D. Bethea, M. M. Elloso, M. Naso and S. Taudte, *Cytokine*, 2011, **53**, 107-114.
3. C. P. Papaneophytou and G. Kontopidis, *Protein Expres. and Purif.*, 2014, **94**, 22-32.
4. B. Wu, S. Muzammil, B. Jones, J. F. Nemeth, D. J. Janecki, A. Baker, M. Merle Elloso, M. Naso, J. Carton and S. Taudte, *Cytokine*, 2014, **65**, 167-174.
5. J. F. Wright, Y. Guo, A. Quazi, D. P. Luxenberg, F. Bennett, J. F. Ross, Y. Qiu, M. J. Whitters, K. N. Tomkinson, K. Dunussi-Joannopoulos, B. M. Carreno, M. Collins and N. M. Wolfman, *J. Biol. Chem.*, 2007, **282**, 13447-13455.
6. H. X. Li, J. Zhang, C. J. An and S. W. Dong, *J. Am. Chem. Soc.*, 2021, **143**, 2846-2856.
7. M. J. Boulanger, D. C. Chow, E. E. Brevnova and K. C. Garcia, *Science*, 2003, **300**, 2101-2104.
8. M. Lebendiker and T. Danieli, *Febs Letters*, 2014, **588**, 236-246.
9. D. K. Ranjit, J. L. Endres and K. W. Bayles, *J. Bacteriol.*, 2011, **193**, 2468-2476.