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Efficient *In Vitro* Refolding and Characterization of Major Histocompatibility Complex Class I-Related Chain Molecules A (MICA) and Natural Killer Group 2 Member D (NKG2D) Expressed in *E. coli*

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Abstract: Major Histocompatibility Complex class I-related chain molecules A (MICA) and receptor Natural killer group 2 member D (NKG2D) are important membrane proteins with immunosurveillance properties which could serve as therapeutic targets for immunotherapy. However, expression of MICA and NKG2D in *E coli* often leads to the formation of inclusion bodies. Here, we present simple, inexpensive and convenient protocol for the solubilization and refolding of inclusion bodies of MICA and NKG2D expressed in *E coli*. The inclusion bodies were firstly dissolved in strong chaotropic reagent (8M urea) and subsequently purified by immobilized-metal affinity column. The denatured MICA/NKG2D was refolded by gradually removing both denaturant (8M urea) and imidazole via dialysis in dialysis buffer of pH 7.4. The appropriate pH of the dialysis buffer was selected based on the theoretical isoelectric points of MICA and NKG2D which were 5.0 and 5.2 respectively. The folded MICA and NKG2D demonstrated the capacity to bind to recombinant NKG2D and MICA respectively by ELISA, Western blot and Surface Plasmon Resonance (SPR) assays. Additionally, the folded MICA and NKG2D demonstrated significant binding to NKG2D-positive Human leukemic cell line U937 and MICA-positive Human pancreatic carcinoma, epithelial-like cell line (PANC-1) respectively, suggesting successful refolding. Successful refolding was further confirmed by Circular Dichroism spectroscopy (CD). We have successfully dissolved, refolded and characterized inclusion bodies of MICA/NKG2D expressed in *E. coli* using simple, inexpensive and convenient protocol which can be carried out in laboratories under-resourced.

Keywords: Inclusion body, MICA, NKG2D, refolding, solubilization, chaotropic reagent.

INTRODUCTION

The heterologous production of membrane proteins in prokaryotic host *E. coli* is a widely used technique in research. However, this technique often leads to the formation of inclusion bodies [1-5]. This has therefore necessitated the need to design protocols by which these inclusion bodies can be solubilized and refolded into biologically active proteins. Major Histocompatibility Complex class I-related chain molecules A (MICA) and Natural killer group 2 member D (NKG2D) are important membrane proteins with immunotherapeutic properties [6, 7]. NKG2D is a homodimeric C-type lectin-like receptor expressed mostly on NK cells and T cells [6, 8]. The NKG2D in association with the adaptor protein DAP10 transduces activating signals which induce cell-mediated cytotoxicity and cytokine secretion [6, 8, 9]. MICA is a stress-inducible protein ligand of NKG2D, which is over-expressed by stressed cells [10]. NKG2D-positive effector cells (NK/T cells) recognize MICA-positive stress cells (tumor cells) via NKG2D-MICA signaling which leads to cytotoxicity of stress cells [11]. In addition, these proteins are reported to be involved in autoimmune diseases [12]. Therefore, successful production of MICA and NKG2D in widely used prokaryotic host *E. coli* would be an important step to ignite interest in the study of these proteins. However, previous attempts to express MICA and NKG2D in *E. coli* led to the formation of inclusion bodies [13, 14]. Although the inclusion bodies of MICA and NKG2D were successfully folded in those previous studies [13, 14], the protocols used seem complex and expensive. In the current study, we used simple, convenient and inexpensive to solubilize and refold the inclusion bodies MICA and NKG2D. Additionally, we used relatively common and inexpensive chemical chaperons which are usually available in almost all laboratories including those under-resourced. This was not the case in the previous attempts where expensive chemical chaperons such as glycine, L-argenine, phenymethylsulfonyl fluoride, sorbitol and sucrose were used in the solubilization and folding of the inclusion bodies [13, 14]. The inclusion bodies of MICA and NKG2D were successfully solubilized in strong chaotropic reagent 8M urea and folded into active conformation by dialysis in dialysis buffer of pH 7.4.

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MATERIALS AND METHODS

Cell culture and Treatment

Human leukemic cell line U937 preserved in our lab was cultured in RPMI 1640 medium, supplemented with 10% (v/v) FBS. Also, Human pancreatic carcinoma, epithelial-like cell line PANC-1 preserved in our lab was cultured in DMEM medium (high glucose), supplemented with 10% (v/v) fetal bovine serum (FBS).

Plasmid Construction of MICA and NKG2D

Genes of the extracellular domains of MICA (residues 1-276) and NKG2D (residues 80-216) without signal peptides were obtained from the GENBANK (accession numbers-MICA: Q29983.1, NKG2D: P267718.1). The genes were then sent to Meiji Co, Ltd. (Shanghai, China) for optimization and synthesis. The synthesized cDNAs were received from Meiji Co, Ltd in the form of the constructions (*C41* (*DE3*)/pET28a-MICA) and (*C41(DE3)*/pET22b-NKG2D). Preferred expression systems *BL21* (*DE3*)/ pET28a-MICA and *BL21* (*DE3*)/pET22b-NKG2D were then constructed in our laboratory. Positive clones were picked from Luria–Bertani (LB) plates containing appropriate antibiotics (100μg/ml Ampicillin for pET22b and 50μg/ml Kanamycin for pET28b) after plating and incubating *BL21*(*DE3*)/ pET28a-MICA and *BL21*(*DE3*)/pET22b-NKG2D for 16-24h. To verify whether the host contains the appropriate cDNA, DNA sequencing was carried out by Meiji Co, Ltd.

Localization of MICA and NKG2D in Different Fractions

Evaluation of the expression location of MICA or NKG2D was done by preparing four cell fractions [15]. Firstly, 200ml culture of MICA or NKG2D was centrifuged at 10.000 rpm for 15 min at 4°C. The supernatant was then collected as secretory expression. Secondly, the residue was suspended in 10ml of 50mM Tris-HCl, 18% sucrose, 0.1mM EDTA (pH 8.0) for 10min and centrifuged at 10,000rpm to collect the supernatant which was named supernatant A. The residue was re-suspended in 10ml hypotonic solution (5mM $MgSO_4$) for 10min and centrifuged at 10,000rpm to collect the supernatant which was named supernatant B. The supernatants A and B were mixed and NaCl added to reach final concentration of 1.5M which was the periplasmic fraction. Thirdly, the cell pellet was re-suspended in 20ml of 50mM cell lysis buffer (50mM Tris-HCl, 0.5MNaCl, 1% TritonX-100 and pH 8.0) and appropriate lysozyme added. After sonication for ten minute, the mixture was then centrifuged at 4℃ for 30 min at 14,000rpm to collect the supernatant as soluble cytoplasmic fraction. Finally, the residue was dissolved in 10 ml of 8M urea buffer for 6h which was the inclusion body fraction. These four different fractions were analyzed by SDS-PAGE, followed by Coomassie blue staining.

Identification of MICA and NKG2D using Western Blot

Western Blotting was used for the preliminary identification of MICA and NKG2D. Protein samples (MICA and NKG2D) 100μg/ml were loaded into 15% (w/v) SDS–PAGE gel for electrophoresis and transferred onto polyvinylidenedifluoride (PVDF) membrane (Millipore). This was carried out for 1.5h in a blotting apparatus (Bio-Rad) under a constant voltage at 100 V. The blotted membrane was placed in blocking buffer TBS (20 mM Tris–HCl, pH 7.4, 150 mM NaCl) with 5% (w/v) skim milk at 37°C for 2h. The membrane was then washed three times with TBS before incubating with His-Tag Mouse mAb (1:2000, Millipore) at 4°C overnight. After washing three times with TBST (TBS containing 0.05 % Tween-20) and TBS, the membrane was incubated with HRP conjugated goat anti-mouse IgG (1:5000, Millipore) at RT for 1.5h. After successive washing as described above, the blots were treated with enhanced chemiluminescence (ECL) solution (Millipore) and exposed in gel imaging systems (Bio-Rad).

Optimization of NKG2D and MICA Expressions

To determine the appropriate temperature that would induce over-expression of MICA or NK2D, expression was induced by the addition of 1.0μmol/ml isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture and incubating at 20, 30or 37°C for 2.5h and shaking at speed 220rpm. To determine the best incubation time, expression was induced by the addition of 1.0μmol/ml IPTG to the culture and incubating for (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 or 6.0h in the case of NKG2D) and (1.0, 3.0, 6.0, 9.0, 12, 15, 18, 20 or 22h for MICA) at 37°C and shaking at speed 220rpm. The suitable concentration of IPTG was determined by adding 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 or 1.6μmol/ml and incubating for 2.5h at 37°C and shaking speed 220rpm.

Expression, Solubilization and Purification of Inclusion Body

Positive clone of MICA/NKG2D was picked and cultured in 10ml LB medium containing (50 μg/ml Kanamycin for MICA)/ (100μg/ml ampicillin for NKG2D) at 37°C overnight. It was then sub-cultured (1:100) in 1L fresh medium containing appropriate antibiotics and incubated (220rpm) at 37°C until the optical density (OD_{600nm}) of the culture reached 0.6. Expression of MICA was then induced by the addition of 1.2mM IPTG and incubating for 20h at 30°C and shaking at speed 180rpm. Expression of NKG2D was induced by the addition of 1.0mM IPTG and incubating for 2.5h at 37°C and shaking at speed 220rpm. Both MICA and NKG2D were expressed mainly as inclusion bodies. As presented in Fig. **1**, the inclusion bodies were processed using the following buffers and reagents; buffer 1 (100mM NaCl, 1mM EDTA, 50mM Tris), buffer2 (100mM NaCl, 10mM EDTA, 1 % Triton), 17.4 mg/ml PMSF and 20mg/ml lysozyme. They were denatured by dissolving in 8M urea at 4°C overnight. Subsequently, they were purified by nickel column chromatography using the following buffers; loading buffer (25mM tris, 500mM NaCl, 8M urea pH 7.5), binding buffer (500ml Na_2HPO_4 8.9535g, NaCl 14.61g, pH 7.5), elution buffer (25mM Tris, 500mM NaCl, 8 M urea, with elution gradient of 0mM, 10mM, 20mM, 50mM, 100mM, 200mM, 400mM, 500 mM imidazole pH 7.5). Successful expression and purification were assessed by Coomassie-stained SDS-PAGE.

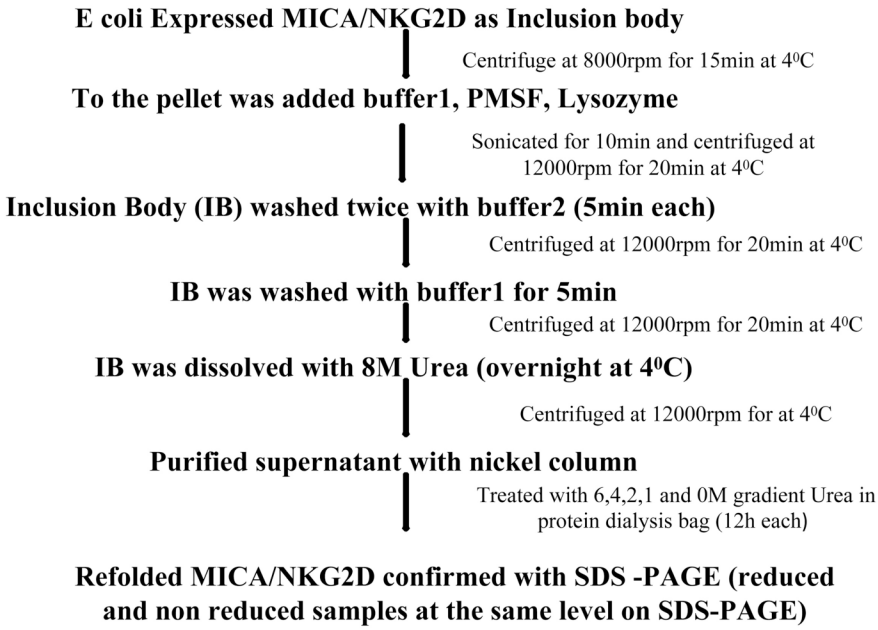

Figure 1. Flow Chart of the Denaturation and Refolding of MICA and NKG2D.

Refolding MICA and NKG2D

The protein sample MICA or NKG2D to be folded was prepared by diluting to a final concentration of 0.3mg/ml. β-Mercaptoethanol (1ml protein sample: 20 µl β-Mercaptoethanol) was then added. Subsequently, it was then dialyzed against decreasing concentration gradient (6, 4, 2, 1 and 0M of urea) in a dialysis buffer (25 mM Tris, 1 mM EDTA, 200 mM NaCl, pH 7.4, 4°C). The dialysis buffer was changed every 12h until the gradient came to its endpoint (0M urea). At concentrations 1 and 0M urea, GSSG (O.4mM) and GSH (3.75mM) were added. Refolded protein was finally dialyzed against PBS (pH 7.4) and preserved for future use. Successful refolding was evaluated by Coomassie-stained SDS-PAGE. The molecular size of the reduced sample (sample with β-Mercaptoethanol) and non-reduced sample (sample without β-Mercaptoethanol) of the refolded target protein were the same, suggesting a successful refolding. Finally, the purity of MICA or NKG2D was assessed by high performance liquid chromatography (HPLC) on an Agilent 1200 system equipped with a TSK-GELG4000PWXL column.

Western Blot Analysis of the Binding Activity of MICA and NKG2D

The binding capacity of refolded NKG2D and MICA was investigated by immunoblotting. NKG2D and MICA (positive control) 100µg/ml were loaded into two different wells on a 15% (w/v) SDS-PAGE gel for electrophoresis and transferred onto polyvinyllidenedifluoride (PVDF) membrane (Millipore). This was carried out for 1.5h in a blotting apparatus (Bio-Rad) under a constant voltage at 100V. The blotted membrane was placed in blocking buffer TBS (20mM Tris–HCl, pH 7.4, 150mM NaCl) with 5% (w/v) skim milk at 37°C for 2h. The membrane was washed three times with TBS and incubated with 0.35mg/ml refolded MICA at 4°C overnight. The membrane was washed three times with TBST (TBS containing 0.05 % Tween-20) and TBS and subsequently incubated with MICA monoclonal antibody (1:2000, Mouse, Code: K0217-3, Clone: AMO1, Concentration: 1mg/ml. Medical & Biological Laboratories Co., LTD) for 2h at 37°C. It was then washed with PBS and incubated with HRP conjugated Goat Anti-Mouse IgG (1:5000, Millipore) at 37°C for 1.5h. After successive washing as described previously, the blots were treated with enhanced chemiluminescence (ECL) solution and exposed in gel imaging system (Bio-Rad).

Quantitative Enzyme Linked Immunosorbent Assay (ELISA)

To confirm the ability of the refolded MICA to bind to recombinant NKG2D and vice versa, quantitative enzyme linked immunosorbent assay was used. Un-treated 96well plates were coated at 100µl/well with 1000mM NKG2D diluted in plating buffer (0.05 M $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. The plating buffer was discarded afterwards and the wells blocked with PBS containing 5% skim milk (200µl/well) and subsequently incubated for another 2h at 37°C. The plates were then washed three times with both PBST (PBS containing 0.05 % Tween-20) and PBS. It was then incubated with serial dilutions of MICA (0.5, 1, 2, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000 nM) for 1.5h at RT. The plates were washed with PBST and PBS and then incubated with MICA antibody mAb (1:2000) diluted in 3% skim milk for 1.5h at RT. After washing as described previously, HRP conjugated Goat Anti-Mouse IgG (1:5000, Millipore) were then added and incubated for further 1.5h at RT. The second antibody (HRP conjugated Goat Anti-Mouse IgG) solution was discarded and the wells washed as described above. Finally, TMB peroxidase sub-

strate (BBI) was added and 50µl of 2.5M sulfuric acid added to the wells to stop the enzyme action. The absorbance was measured at OD_{450}-OD_{630}.

Binding Kinetic Analysis of MICA and NKG2D

The binding kinetics of refolded MICA and NKG2D were measured with a Biacore X100(GE) instrument (Biacore X100, GE Healthcare, Sweden) at 25°C. The running buffer (10mM HEPES, 150mM NaCl, 0.005% Surfactant polysorbate20, 50µM EDTA at pH7.4) and the dispensor buffer (10mM HEPES, 150mM NaCl, 0.005% Surfactant polysorbate 20, 3mM EDTA) were filtered and degassed prior to their usage. Firstly, MICA was diluted in the running buffer and immobilized on Sensor Chip CM5 (GE Healthcare, BR-1000-12) with target resonance unit (RU) density of 2000. NKG2D (250, 125, 62.5, 31.5, 15.6, 7.8nM) was then injected at different concentrations. The capture was done at a constant flow rate of 40µl/min. Sensorgrams were obtained at each concentration and evaluated using the BIA Evaluation 2.0 program. The kinetic constants association (k_a), dissociation (k_d), and equilibrium constant (K_D) calculated with 1:1 binding model.

Flow Cytometry Analysis of the Affinity Between Refolded MICA and NKG2D

To determine whether refolded MICA and NKG2D can bind to native NKG2D and MICA respectively, the flow cytometry assay was carried out. NKG2D-positive U937 and MICA-positive PANC-1 cells were used. 5×10^5 of U937 and PANC-1 cells per sample were suspended in PBS containing 5% BSA and then incubated with 2000mM MICA and 2000mM NKG2D at 4°C for 1h respectively. The cells were then incubated with His-probe (H-15) rabbit (sc-803) or goat (sc-803-G) poly-clonal affinity purified antibody (Santa Cruz Biotechnology). The cells were subsequently washed and biding assay carried out with a BD FACS flow cytometer. PBS was used as control. The refolded MICA and NKG2D were subsequently characterized by Circular dichroism spectroscopy using MICA (12302-H08H-50) and NKG2D (10575-H07B-50) purchased form Sino biological inc. as standard samples.

RESULTS

Localization of MICA and NKG2D in Different Fractions

MICA and NKG2D were mainly expressed as inclusion bodies (Fig. **2A** and **B**). However, the other fractions secretory protein, periplasmic protein and cytoplasmic protein as shown in Fig. **2A** and **2B** were not over-expressed. Expression of MICA and NKG2D was confirmed by western blot analysis (Fig. **2C**). These data confirmed that both MICA and NKG2D are expressed mainly as inclusion bodies in *E coli*.

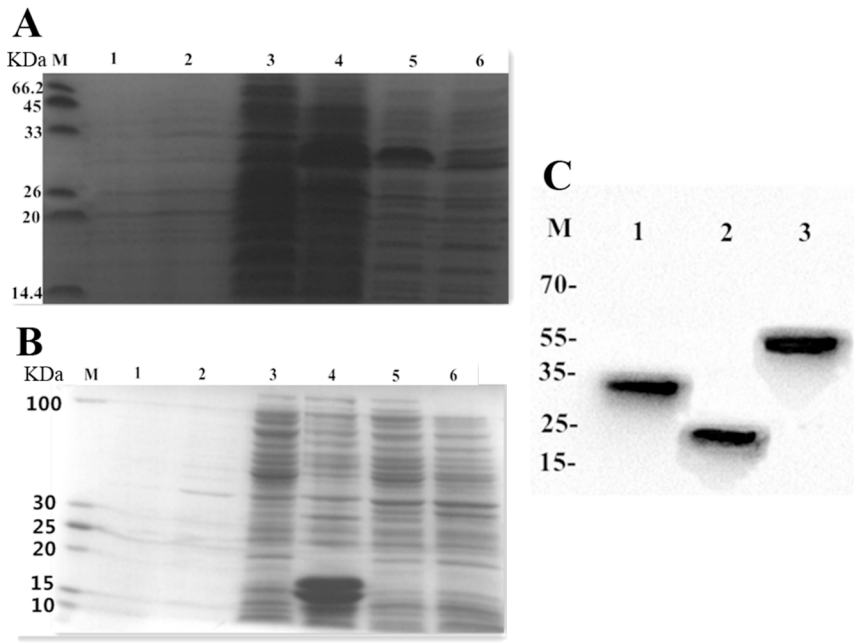

Figure 2. Coomassie-stained SDS-PAGE analysis of four fractions of *BL21(DE3)*/pET28a-MICA and *BL21(DE3)*/pET22b-NKG2D expressions, and Western blot analysis of a successful expression of MICA and NKG2D. (**A**) Coomassie-stained SDS-PAGE analysis of four fractions of *BL21(DE3)*/pET28a-MICA expression. Lane 1: Secretary expression; Lane 2: Periplasmic expression; Lane 3: Cytoplasmic expression; Lane 4: Inclusion body; Lane 5: Whole cell; Lane 6: Negative. (**B**) Coomassie-stained SDS-PAGE analysis of four fractions of *BL21(DE3)*/pET22b-NKG2D expression. Lane 1: Secretary expression; Lane 2: Periplasmic expression; Lane 3: Cytoplasmic expression; Lane 4: Inclusion body; Lane 5: Whole cell; Lane 6: negative. (**C**) Western blot analysis of a successful expression of MICA and NKG2D. Lane 1: MICA expression (32 KDa) detected using Western blot; Lane 2: NKG2D expression (16 KDa) detected using Western blot; Lane 3: Positive control.

Optimization of NKG2D and MICA Expression

As shown in Fig. **3**, the appropriate conditions for the over-expression of MICA in *E coli* are 1.2μmol/ml, 20h, 30°C and shaking at speed 180rpm. The favourable conditions for the over-expression of NKG2D in *E coli* are 1μmol/ml IPTG, 37°C, 2.5h and shaking at speed 220rpm (Fig. **4**). The data further confirmed that MICA and NKG2D are mainly expressed as inclusion bodies in *E coli* and that they can be over-expressed by employing the conditions stated above.

Denaturation, Purification and Refolding of Inclusion Bodies

The inclusion bodies of MICA (Fig. **5A** and **B**) and NKG2D (Fig. **5D** and **E**) were successfully solubilized, purified and refolded. Successful refolding was initially evaluated by Coomassie-stained SDS-PAGE. The molecular weight of the reduced sample (sample treated with β-Mercaptoethanol) and non-reduced sample (sample without β-Mercaptoethanol) of target proteins were the same, suggesting that both MICA and NKG2D were successfully refolded. Also, the HPLC analysis (Fig. **5C** and **F**) showed that MICA and NKG2D were more than 90% pure.

ELISA and Western Blot Analysis of the Affinity Between MICA and NKG2D

These assays were carried out to confirm successful refolding by assessing whether the refolded MICA and NKG2D can bind to recombinant NKG2D and MICA respectively. The ELISA analysis showed that the refolded

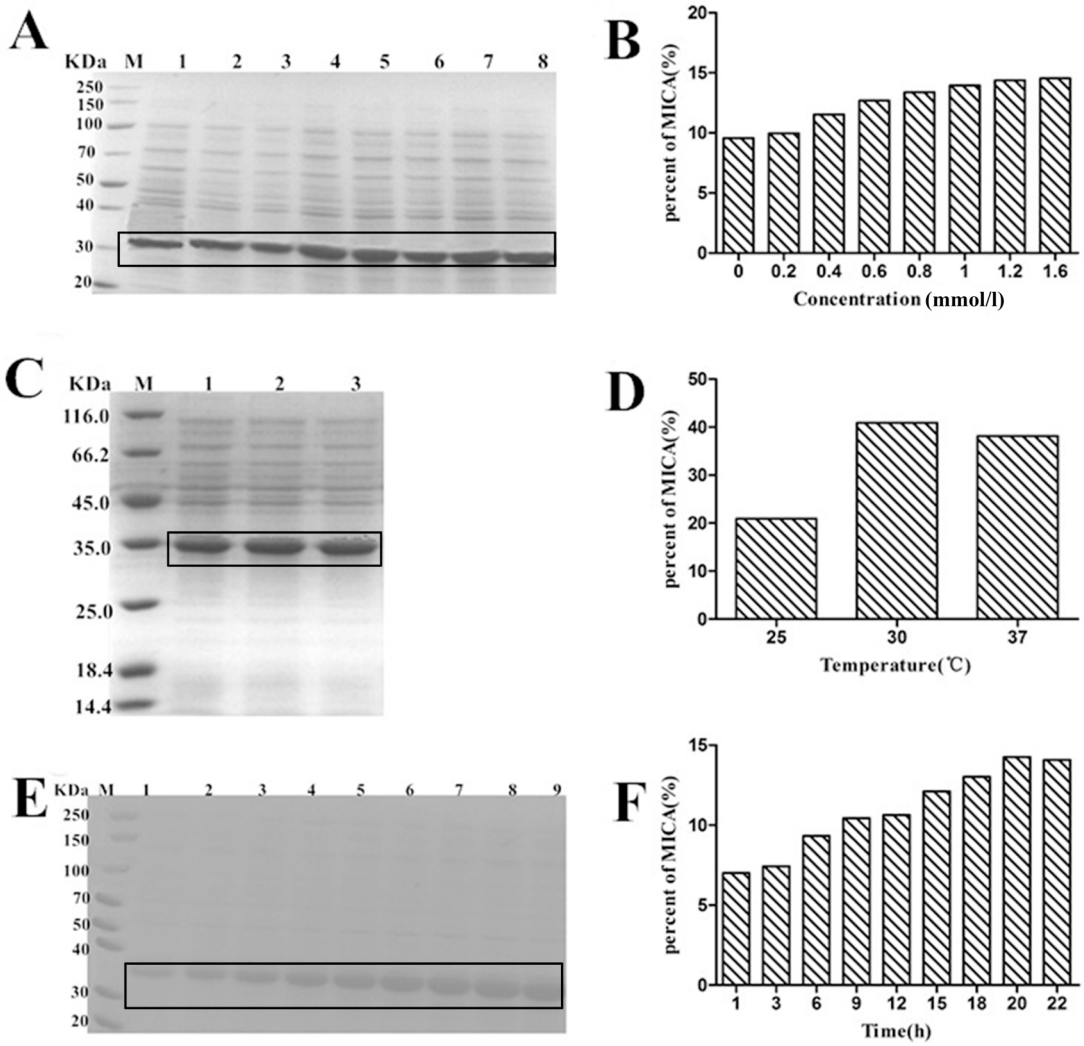

Figure 3. Optimization of the expression of MICA. (**A**) Coomassie-stained SDS-PAGE analysis of expression of MICA with different IPTG concentrations. Lane M: Marker; Lane 1-8: MICA expression at different IPTG concentrations. (**B**) Quantification of MICA expression at different IPTG concentration. The results demonstrate that over-expression of MICA can be achieved at 1.6 mol/L IPTG. (**C**) Coomassie-stained SDS-PAGE analysis of expression of MICA at different temperatures. Lane M: Marker; Lane 1-3: MICA expression at different temperatures. (**D**) Quantification of MICA expression at different temperatures. The results demonstrate that over-expression of MICA can be achieved at 30°C. (**E**) Coomassie-stained SDS-PAGE analysis of expression of MICA at different times (duration). Lane M: Marker; Lane 1-9: MICA expression at different times (duration). (**F**) Quantification of MICA expression at different times (durations). The results demonstrate that over-expression of MICA can be achieved at 20h.

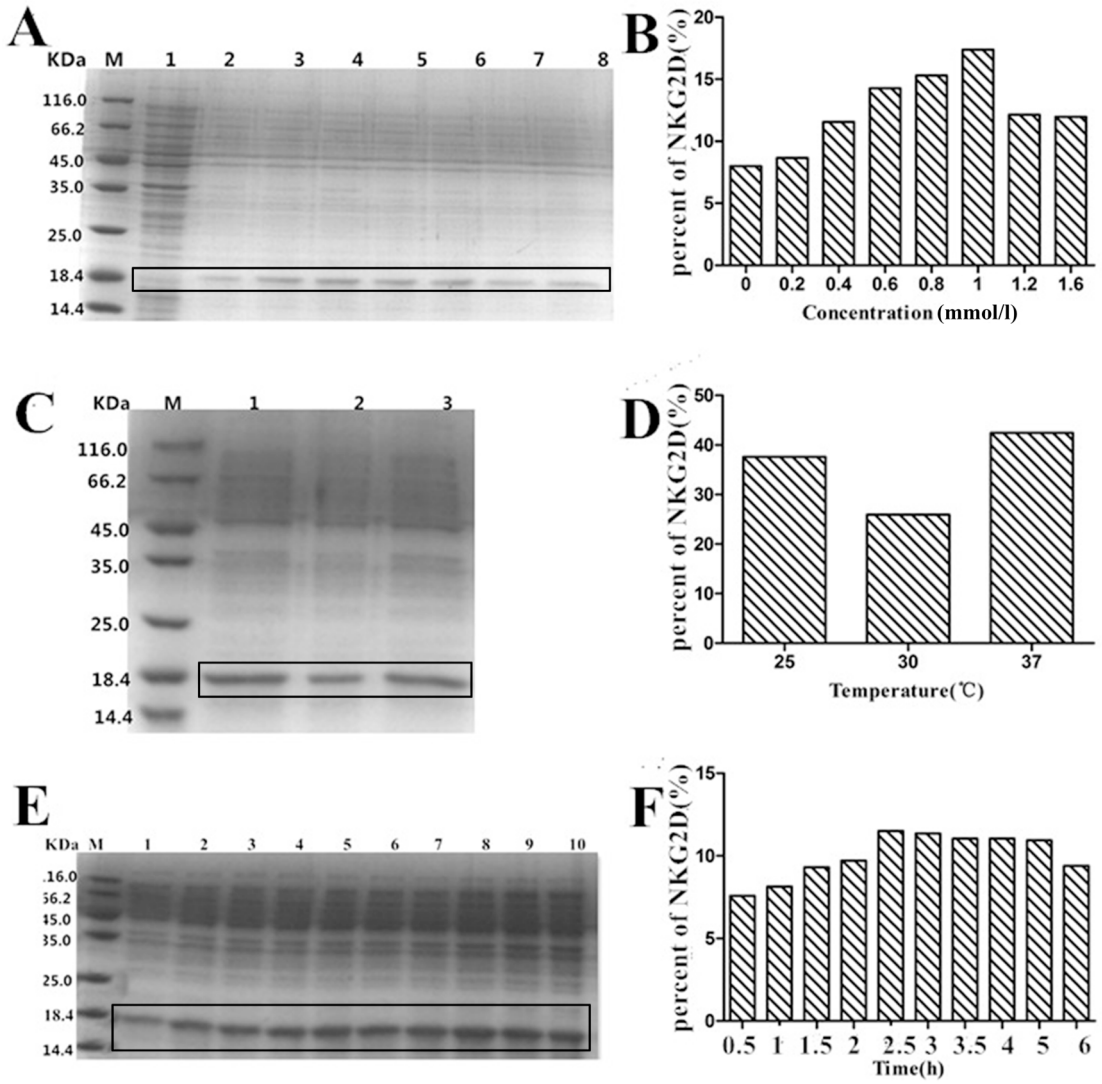

Figure 4. Optimization of the expression of NKG2D. (**A**) Coomassie-stained SDS-PAGE analysis of expression of NKG2D with different IPTG concentrations. Lane M: Marker; Lane 1-8: NKG2D expression at different IPTG concentrations. (**B**) Quantification of NKG2D expression at different IPTG concentrations. The results demonstrated that over-expression of MICA can be achieved at 1mol/L IPTG. (**C**) Coomassie-stained SDS-PAGE analysis of expression of NKG2D at different temperatures. Lane M: Marker; Lane 1-3: NKG2D expression at different temperatures. (**D**) Quantification of NKG2D expression at different temperatures. The results demonstrate that over-expression of NKG2D can be achieved at 37°C. (**E**) Coomassie-stained SDS-PAGE analysis of expression of NKG2D at different times (duration). Lane M: Marker; Lane 1-10: NKG2D expression at different times (duration). (**F**) Quantification of MICA expression at different times (durations). The results demonstrated that over-expression of MICA can be achieved at 2.5h.

MICA can dose dependently bind to the refolded NKG2D (Fig. **6A**). Furthermore, their binding capacities were confirmed by western blot analysis. Refolded MICA demonstrated significant binding to the refolded receptor NKG2D (Fig. **6B**). It can therefore be inferred that both MICA and NKG2D were successfully refolded and as such could be used for bioactivity studies.

Binding Kinetic Analysis of MICA and NKG2D

The binding kinetics between the refolded MICA and the refolded NKG2D was determined by SPR technology on a BIAcore instrument and evaluated with the BIA-evaluation program. The calculated value of the dissociation constant K_D ($K_D=k_d/k_a$) between MICA and NKG2D was 3.95×10^{-8}M (Fig. **6E**.) where $k_a= 7.02\times10^{-7}$/MS and $k_d= 4.03\times10^{-13}$/S. The results demonstrated that, the association rate increased with increasing concentration of NKG2D. The whole kinetic process can therefore be described as quick association and slow dissociation. These further confirmed the binding affinity between MICA and NKG2D and the fact that MICA and NKG2D were successfully refolded.

Flow Cytometry Analysis of the Affinity Between MICA and NKG2D

This assay was carried out to determine the ability of MICA and NKG2D to bind to native NKG2D and MICA

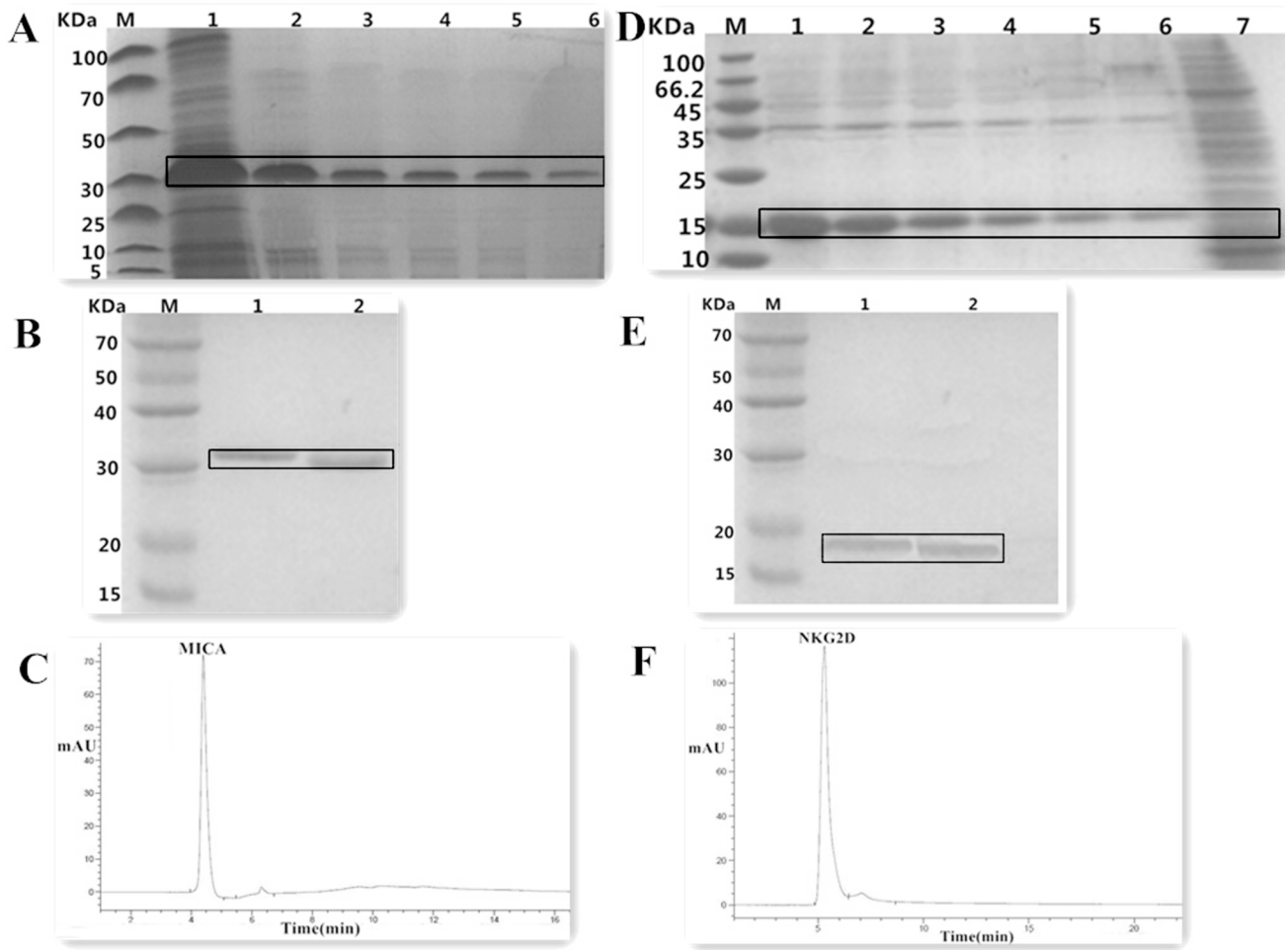

Figure 5. Coomassie-stained SDS-PAGE and HPLC analyses of purified and refolded MICA and NKG2D. (**A**) Coomassie-stained SDS-PAGE analysis of purified MICA. Lane M: Marker; Lane 1: Unpurified MICA sample; Lane 2-6: Different elution collections of purified MICA. (**B**) Coomassie-stained SDS-PAGE analysis of purified refolded MICA. Lane M: Marker; Lane 1: Reduced sample of MICA (MICA treated with β-Mercaptoethanol); Lane 2: Non-reduced sample of MICA (MICA without β-Mercaptoethanol). (**C**) HPLC analysis of refolded MICA. (**D**) Coomassie-stained SDS-PAGE analysis of purified NKG2D. Lane M: Marker; Lane 1-6: Different elution collections of purified NKG2D; Lane 7: Unpurified NKG2D sample. (**E**) Coomassie-stained SDS-PAGE analysis of purified refolded NKG2D. Lane M: Marker; Lane 1: non-reduced sample of NKG2D (NKG2D without β-Mercaptoethanol); Lane 2: Reduced sample of NKG2D (NKG2D treated with β-Mercaptoethanol). (**F**) HPLC analysis of refolded NKG2D.

respectively. As shown in Fig. **6C**, refolded MICA demonstrated significant binding to NKG2D-positive U937 cells. Additionally, refolded NKG2D demonstrated significant binding to MICA-positive PANC-1 cells (Fig. **6D**). These data further demonstrated that both MICA and NKG2D were successfully folded and therefore could be used for bioactivity studies. Additionally, the efficiency of the refolding process used in this study was evaluated by characterizing the refolded proteins MICA and NKG2D through Circular dichroism spectroscopy, using commercially produced MICA (12302-H08H-50) and NKG2D (10575-H07B-50) from Sino biological inc. as standard samples. As shown in fig. **7**, our refolded MICA and NKG2D demonstrated similar secondary conformation to the MICA and NKG2D purchased from Sino biological inc. respectively, suggesting successful refolding.

DISCUSSION

Escherichia coli is one of the most widely used organisms for recombinant DNA technology since the 70s because of its robust nature. However, the expression of membrane proteins in *E. coli* often leads to the formation of dense insoluble particles called inclusion bodies [16]. Inclusion bodies are believed to be aggregates of polypeptide chains which have been partly or completely misfolded. As reported elsewhere [13, 14], MICA and NKG2D are mainly expressed as inclusion bodies in *E. coli*. An efficient protocol is therefore required to dissolve and refold the inclusion bodies into bioactive conformation before they can be used for bioactivity studies. In the current study, we sought to denature, purify and refold the inclusion bodies of MICA and NKG2D expressed in *E. coli* with inexpensive and convenient protocol. Contrary to the protocol used in previous studies elsewhere

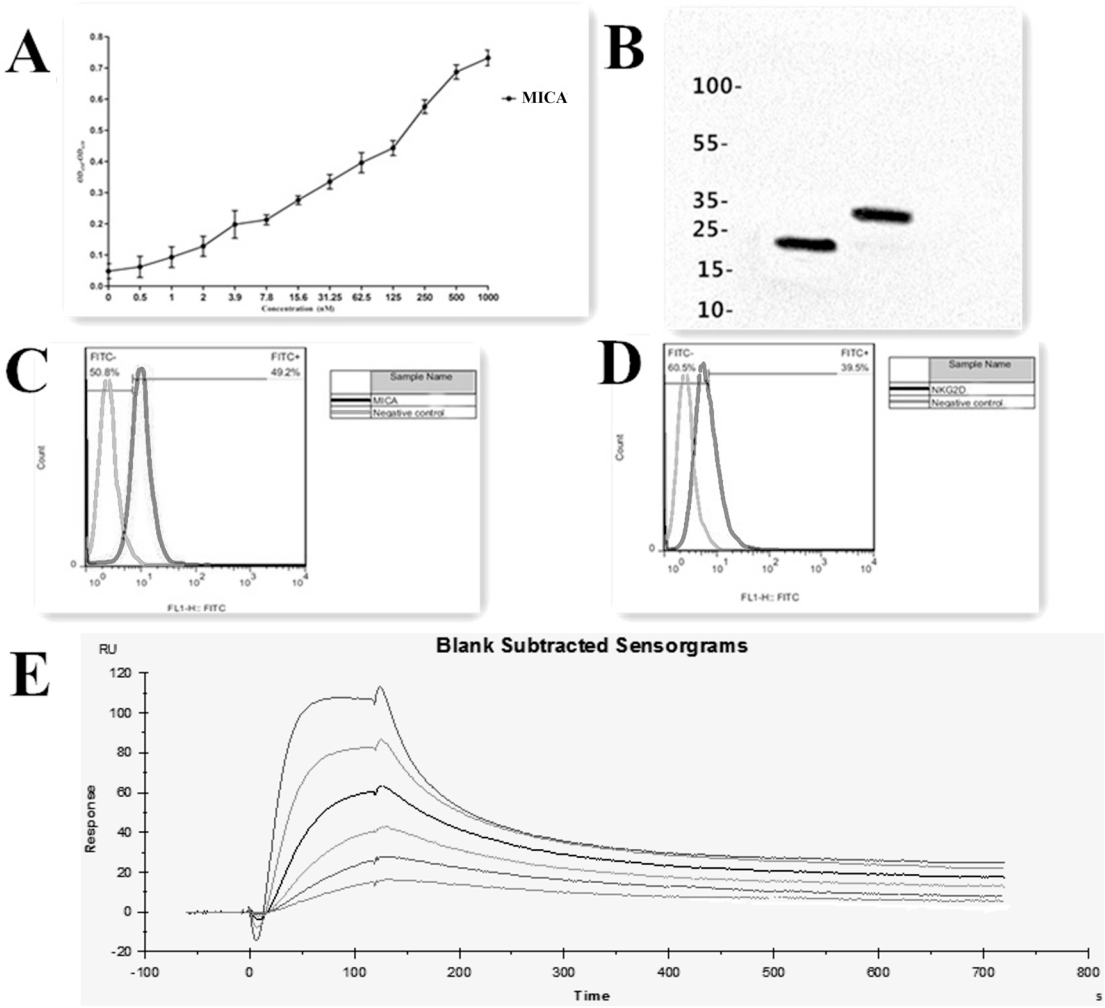

Figure 6. Determination of ability of refolded MICA to bind to NKG2D vice versa using Western blot, ELISA, SPR and flow cytometry assays. (**A**) Binding between refolded MICA and NKG2D detected by ELISA. As the results showed, MICA demonstrated dose-dependent binding to NKG2D. (**B**) Binding between refolded MICA and NKG2D detected by Western blot. Lane 1: MICA binds to NKG2D (16 KDa), suggesting a successful refolding of both MICA and NKG2D. Lane 2: positive control MICA (32 KDa). (**C**) MICA demonstrated significant binding to NKG2D-positive U937 cells with binding rate 49.2%. On the contrary, PBS (negative control) demonstrated no significant affinity to NKG2D-positive U937 cells. (**D**) NKG2D exhibited significant binding ability to MICA-positive PANC-1 cells with binding rate 39.5%. However, PBS (negative control) demonstrated no significant binding ability to MICA-positive PANC-1 cells. (**E**) SPR sensorgrams of the binding kinetics of refolded MICA against refolded NKG2D. The calculated value of the dissociation constant (K_D) ($K_D=k_d/k_a$) of MICA and NKG2D was ($k_a= 7.02\times10^{-7}$/MS and $k_d= 4.03x10^{-13}$/S) 3.95×10^{-8}M.

[13, 14] to denature and refold inclusion bodies of MICA and NKG2D, we used relatively simple, inexpensive and convenient refolding protocol. In addition, unlike the previous studies [13, 14] where relatively expensive chemical chaperons such as glycine, L-argenine, phenymethylsulfonyl fluoride, sorbitol and sucrose were used, we used relatively inexpensive and common chemical chaperons which can be available in almost all laboratories including the under-resourced. The inclusion bodies were isolated from *E. coli* lysate and washed. Washing of the inclusion bodies was done to improve the final yield of the refolded proteins. The inclusion bodies were then dissolved in strong chaotropic reagent (8M urea). This was done to disrupt the hydrogen bonding network between the water molecules thereby weakening the hydrophobic effect and increasing the solubility of the target proteins MICA and NKG2D [16]. The temperature was kept at 4^{o}C during the process to prevent the formation of cyanate which could lead to carbamylation of the amino groups. Because the target protein is a His-tag fusion construct, purification of the solubilized protein was done by immobilized metal affinity chromatograpohy in the presence of strong chaotropic reagent 8M urea [17]. It was imperative to purify MICA and NKG2D in the presence of 8M urea in order to prevent the re-formation of aggregates after successfully dissolving the inclusion bodies. The protein was subsequently dialyzed against a decreasing concentration of the chaotropic reagent (6, 4, 2, 1 and 0M Urea) to gradually remove the chaotropic reagent. The chaotropic reagent was gradually removed to facilitate proper folding of the dissolved inclusion bodies, since it reduces aggregation.

The sample to be refolded was diluted to a final concentration of 0.3 mg/ml. This concentration was selected, after

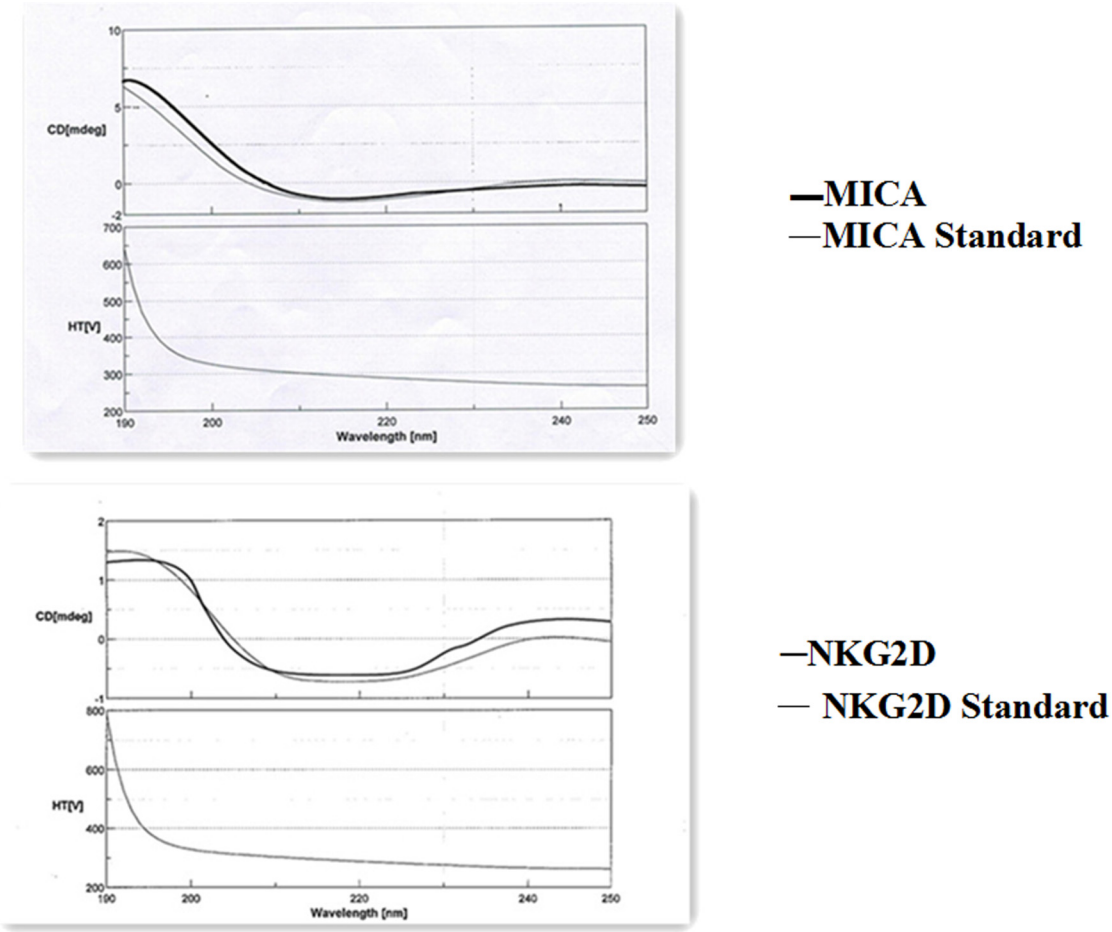

Figure 7. Circular dichroism spectroscopic (CD) analysis of the refolded proteins. MICA and NKG2D showed similar characteristics as the commercially purchased MICA (12302-H08H-50) and NKG2D (10575-H07B-50). This shows that MICA and NKG2D were successfully refolded and that the protocol used in this study is efficient.

thoroughly screening to select best concentration that yields the optimum folding. Additionally, due to the fact that our target proteins contain cysteine residues which could possibly facilitate the formation of non-native, inter- and intramolecular disulfide bonds during the inclusion bodies formation [18-19], samples were firstly treated with reducing agent β-Mercaptoethanol before commencing the refolding process. Also, reduced and oxidized glutathione (GSSG and GSH) were added at the later stages of the dialysis (at 1 and 0 M Urea) to promote the generation of correct disulphide bond configuration in the folded protein [20]. EDTA was also added to the refolding buffer to prevent oxidative side reactions during the refolding process. Appropriate dialysis bag for refolding is usually selected based on the molecular weight of the protein to be refolded. In the current study, dialysis bag sizes MWCO 10K. W 34mm and MWCO 5 K. W 34mm (The American association of spectrum) were selected for MICA (32 KDa) and NKG2D (16 KDa) respectively.

The pH of the dialysis buffer [21] plays very important role in the success of the folding process. The pH of the dialysis buffer in this study was selected based on the theoretical isoelectric point (PI) of MICA and NKG2D. The pH of the dialysis buffer should usually be at least 1 unit away from the PI so that the protein molecules will have net charges. This would ensure that they repel each other to prevent them from sticking together and re-aggregating. The theoretical PI of MICA and NKG2D are 5.2 and 5.0 respectively and therefore pH of the dialysis buffer was kept at 7.4. Under reducing condition, SDS-PAGE can discriminate between aggregates held together by disulfide bonds and those held together by non-reducible covalent bonds [22]. Successful folding of MICA/NKG2D was therefore evaluated by SDS-PAGE in this study. This was done by analyzing the reduced and non-reduced samples of MICA/NKG2D to differentiate between disulfide bond which is reducible and non-reducible covalent bonds. As shown on Fig. **5**, sample of MICA/NKG2D treated with reducing agent β-Mercaptoethanol had the same molecular size as the MICA/NKG2D sample without β-Mercaptoethanol. This demonstrates that refolded MICA and NKG2D were without reducible disulfide bonds responsible for the formation of inclusion bodies, suggesting that the refolding process was successful. However, further experiment will be needed to evaluate whether the refolded MICA and NKG2D were monomeric proteins or aggregates. Successful refolding of the inclusion bodies of MICA and NKG2D were confirmed by assessing their binding capacity using ELISA, SPR, Western blot and flow cy-

tometry assays. The ability of the refolded MICA to bind to folded NKG2D was evaluated by ELISA, SPR and Western blot assays. And as shown in Fig. **6**, MICA and NKG2D demonstrated significant binding to each other. Additionally, the ability of the refolded MICA and NKG2D to bind to native NKG2D and MICA respectively was evaluated by flow cytometry. And as shown in Fig. **6**, refolded MICA demonstrated specific binding to NKG2D-positive U937 cells [23]. Also, refolded NKG2D demonstrated specific binding to MICA-positive PANC-1 cells. It can therefore be inferred that MICA and NKG2D have been refolded successfully and that they could be used for bioactivity studies. Additionally, the refolding protocol in this study was further evaluated by comparing the refolded MICA and NKG2D to their counterparts (MICA and NKG2D) produced by Sino biological inc. using Circular dichroism spectroscopy. And as the results (Fig. **7**) depicted, our refolded MICA and NKG2D shared similar secondary conformational characteristics with the MICA and NKG2D purchased from Sino biological inc. respectively, demonstrating proper refolding. This implies that our new protocol is efficient although inexpensive and convenient.

In summary, we have efficiently solubilized, purified and refolded the inclusion bodies of MICA and NKG2D expressed in *Escherichia coli* and demonstrated that they can be used for biological studies. This study therefore presents simple, inexpensive and convenient protocols for the solubilization, purification and folding of inclusion bodies, which can be repeated in any averagely equipped laboratory underresourced.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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