# Isolation and characterization of a novel nanobody for detection of GRP78 expressing cancer cells

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## Abstract

Glucose-regulated protein 78 (GRP78) is an endoplasmic reticulum (ER) chaperone that has been shown that is overexpressed in cancer cells. Overexpression of GRP78 on cancer cells makes this molecule a suitable candidate for cancer detection and targeted therapy. VHH is the binding fragment of camelid heavy-chain antibodies also known as "nanobody." The aim of this study is to isolate and produce a new recombinant nanobody using phage display technique to detect cancer cells. Using the c-terminal domain of GRP78 (CGRP) as an antigen, four rounds of biopanning were performed, and high-affinity binders were selected by ELISA. Their affinity and functionality were characterized by surface plasmon resonance (SPR) cell ELISA and immunocytochemistry. A unique nanobody named V80 was purified. ELISA and SPR showed that this antibody had high specificity and affinity to the GRP78. Immunofluorescence analysis showed that V80 could specifically bind to the HepG2 and A549 cancer cell lines. This novel recombinant nanobody could bind to the cell surface of different cancer cells. After further evaluation, this nanobody can be used as a new tool for cancer detection and tumor therapy.  Volume 68, Number 2, Pages 239–246, 2021



***Keywords:*** *glucose-regulated protein 78, Camelidae antibody, cancer detection, nanobody, unfold protein response*

***Abbreviations:*** *CGRP, c-terminal domain of GRP78; GRP78, glucose regulated protein 78; sdAB, single domain antibody; SPR, surface plasmon resonance; UPR, unfold protein response.*

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## 1. Introduction

The endoplasmic reticulum (ER) is a subcellular organelle responsible for proper folding of membrane and secretory proteins. Under stress conditions, where the rate of unfolded/misfolded proteins is high and beyond ER folding capacity, unfolded protein response (UPR) is activated. Glucose-regulated protein 78 (GRP78) is a highly abundant ER chaperon that binds to hydrophobic regions of misfolded or unfolded proteins and promotes their proper folding. GRP78 is one of the main signaling cascade components leading to UPR. When the misfolded proteins are accumulated in the ER lumen, GRP78 is dissociated from mediators, leading to their activation and UPR promotion [1].

Either due to intrinsic or extrinsic conditions, including acidosis, hypoxia, nutrient deprivation, and hypoglycemia in tumor microenvironment, tumor cells are subjected to stress and UPR activation [2, 3].

As one of the main components in UPR, GRP78 expression in cancer cells is higher than in normal cells. The overexpression of GRP78 is reported in various cancers, including breast cancer [4], hepatocellular carcinoma [5], lung cancer [6], and prostate cancer [7]. It is shown that GRP78 expression level in cancer tissues depends on the tumor stage [8] and is associated with resistance to chemotherapy [9] and apoptosis suppression [4, 10].

After the first report in 1997 [11], evidence for GRP78 localization on the cancer cell surface has grown rapidly [12]. GRP78 cell surface localization is reported in pancreatic cancer cells, osteosarcoma, hepatoma, melanoma, and breast cancer [13-15].

Overexpression of GRP78 on cancer cells, provides an opportunity to detect and target cancerous cells [16]. A monoclonal antibody directed toward carboxyl terminal domain of GRP78 (CGRP)-induced apoptosis in 1-LN, DU145, and A375 cancer cell lines [17]. The results of the above studies indicate that C- terminal of GRP78 is a suitable candidate to be targeted via antibodies.

In addition to conventional antibodies, Camelidae have a unique class of antibodies consisting of only the heavy chains, called heavy-chain antibodies (HcAb). The amino terminal region of the variable domain of HcAb or VHH is capable of independent antigen recognition and binding [18]. Recombinant expression of VHH called single domain antibody (sdAb) or nanobody Due to unique features, including small size (approximately 15 kDa), high expression in various hosts, low immunogenicity in human, high solubility and stability, high penetration to different tissues, and easiness of conjugation with various agents, nanobodies are considered as a new attractive agent in cancer detection and therapy [19, 20]. In this study, we developed a new VHH antibody directed against GRP78 by phage display method. The potency of this nanobody to bind to GRP78 and detect cancer cells was evaluated.

**Highlights**

- We prepared the first nanobody against CGRP.
- Anti-GRP78 nanobody showed high specificity and reasonable affinity.
- This nanobody can detect cancer cells.

## 2. Material and Methods

### 2.1. Preparation of CGRP

Cloning and recombinant expression of CGRP were reported in our previous study [21]. A 690bp sequence of the human *CGRP* gene encoding amino acids 150–380 was synthesized (Biomatik, Ontario,Canada), subcloned into the pET22b(+) vector (Novagen, Gibbstown, USA) and expressed in *Escherichia coli* T7 shuffle cells. Expression was induced by 0.5mM isopropyl-$\beta$-D-thiogalactoside (IPTG) at 30°C overnight. The expression of recombinant CGRP was analyzed by SDS-PAGE and purified by affinity chromatography using Ni-NTA resin (Qiagen, Venlo, The Netherlands). The protein was confirmed by ELISA using anti-CGRP antibody (Abcam,Cambridge, United Kingdom).

### 2.2. Biopanning of phage-VHH against CGRP

Different VHH libraries from our previous studies [22-25] and a library from Camelus dromedaries immunized with human cancer tissues as described by Sharifzadeh et al. [26] were mixed.

For biopanning, 2μg of CGRP in 100μL of the bicarbonate coating buffer (0.05M carbonate-bicarbonate, pH 9.6) was coated in a well of 96-well plates. Bovine serum albumin (BSA) was coated as the control. After washing with 200μL phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST), the wells were blocked with 5% (w/v) BSA in PBS. After second washing with PBST, 100μL of the phage-VHH library was added to each well, and the plate was incubated at 37°C for 1H with shaking. To remove the unbound or weakly bound phage particles, the wells were washed 10 times with PBST. Bound phages were eluted with 100μL of 0.25mg/mL trypsin solution. For amplification, 5mL of exponentially growing *E. coli* TG1 cells were inoculated with 50μl of the eluted phage and incubated for 30Min at 37°C. Then, 7mL of LB culture medium supplemented with 100μg/mL ampicillin and 2% (wt/vol) glucose was added to the culture and incubation continued overnight at 37°C with shaking. To rescue and amplify phage particles, 50mL of 2×YT medium supplemented with 100μg/mL ampicillin and 2% (wt/vol) glucose was inoculated with 500μL of the overnight culture in a baffled 250-mL Erlenmeyer flask. The cells were incubated at 37°C until they reached the exponential growth phase. After super infection of the cells with M13K07 helper phage (Amersham-Pharmacia-Biotech, Little Chalfont, United Kingdom) and

supplementation of culture with kanamycin (50μg/mL) and ampicillin (100μg/mL), the flask was incubated overnight at 37°C with shaking at 200rpm. Following centrifugation at 5,000*g* for 10Min, 40mL of the supernatant was transferred to a new Falcon tube and phage particles were precipitated using 20% Polyethylene glucol (PEG)/ Sodium chloride (NaCl) (*v/v*). The obtained phages were then titrated and used in the next rounds of panning [27]. Four rounds of panning were performed.

*2.2.1. Biopanning analysis by polyclonal phage ELISA*

An ELISA reaction was performed to check the biopanning process. For each panning round, two wells of a microtiter ELISA plate were coated with 100 μL of 20 μg/mL CGRP suspended in the bicarbonate coating buffer. After blocking with 5% BSA, phage particles from each biopanning round were added. Then, 100 μL of anti-M13 antibody conjugated to horseradish peroxidase (HRP) (Amersham, Little Chalfont, United Kingdom) at a 1:5,000 dilution was added to each well and incubated at 37 °C for 30 min. Tetramentylbenzidine (TMB), as a chromogenic substrate, was added to each well. The reaction was stopped by 2 M $H_2SO_4$, and the $OD_{450}$ was measured.

### 2.3. Selection of phage antibody

The highest $OD_{450}$ was observed at the first round of panning, therefore 300 clones were randomly selected from this round, and a few clones were picked from other rounds. After overnight incubation, 10μL of the culture was inoculated to a well of a 96–well plate containing 2×YT medium supplemented with ampicillin (100μg/mL). The plate was incubated at 37°C and 150rpm until reaching the exponential growth phase. Nanobody expression was induced by adding IPTG with the final concentration of 1mM. Colonies expressing nanobody with the highest affinity to CGRP were selected by ELISA. To conduct the ELISA, 2μg of CGRP was coated in a 96-well plate. Then, 100μL of nanobody expressing cell lysate was added, and wells were washed three times with PBST. Hundred microliters of mouse anti-HA antibody (Genescript, New Jersey, USA) was used as primary antibody (1:5,000), and HRP-conjugated (1:30,000) anti-mouse antibody (Sigma, Missouri, USA) was used as the secondary antibody. Detection was performed using TMB. Colonies expressing high-affinity nanobodies were sequenced.

### 2.4. Cloning, expression, and purification of selected nanobody

DNA fragment encoding VHH from the highest binder (V80) was amplified using PCR. This fragment was ligated using *Eco*RI and *Xho*I restriction sites of pET28a(+) expression vector. The *pET-VHH* construct was transferred to *E. coli* BL21(DE3) by electroporation. Protein expression was induced by 0.5 mM IPTG and incubation at 30 °C overnight. Bacterial cells were collected by centrifugation and resuspended in PBS. The cells were lysed using sonication and after centrifugation, the supernatant was loaded on a Ni-NTA affinity column (Qiagen, Venlo, The Netherlands). The column was washed using buffers with sequentially increasing concentrations of imidazole (20–100 mM). VHH was finally eluted from the resin using the elution buffer containing 250 mM imidazole. The purity of VHH was analyzed using SDS-PAGE.

### 2.5. Specificity determination

The cross-reactivity of V80 with several nonspecific proteins, including B-lymphocyte antigen CD20, tumor necrosis factor, interleukin-8, and diisopropyl fluorophosphatase was evaluated via ELISA. These proteins were available in our laboratory and used as antigens in specificity tests. The ELISA plate wells were coated with the aforementioned proteins. GRP78 (Abcam, Cambridge, United Kingdom) was used as positive control. Prior to incubation with 100 μL (20 μg/mL) of V80, the wells were blocked as mentioned before. Detection of bound nanobodies was performed by anti-HA (Genescript, New Jersey, USA) and anti-mouse HRP-conjugated IgG (Sigma, Missouri, USA) as primary and secondary antibodies, respectively.

### 2.6. Affinity calculation by surface plasmon resonance

Interactions of the nanobody with GRP78 were studied by means of a two-channel cuvette-based surface plasmon resonance (SPR) system (Metrohm Autolab, Utrecht, the Netherlands). After formation of a 11-mercaptoundecanoic acid self-assembled monolayer on sensor chip and activation of carboxyl groups by a mixture of freshly prepared1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (400mM) and *N*-Hydroxysuccinimide (NHS) (100mM) for 300Sec, GRP78 (50μg/mL) was immobilized on the sensor surface by the amine coupling method. To assess $K_D$, different concentrations of nanobody (25–400nM) interacted with immobilized GRP78. The association step was performed for 900Sec, and dissociation was performed for 600Sec. To bring the signal to the baseline level to start a new measurement cycle, the target bounded GRP78 was recovered by an optimized regeneration buffer (acetate buffer+SDS 0.5%) after each measurement. All interactions were performed at 25°C in HBS buffer [10mM(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HEPES pH 7, 150mM NaCl, 3mM (Ethylenediaminetetraacetic acid) EDTA, and 0.05% Tween-20]. Unimmobilized channel was used as an online reference during all the binding experiments [28]. $K_D$ was evaluated using the "kinetic evaluation software ver. 5.4" (Kinetic Evaluation Instruments BV,Leusden, The Netherlands). The data were fitted using a simple 1:1 Langmuir fit model.

### 2.7. Cell ELISA

A549 (ATCC CCL-185) and HepG2 (ATCC HB-8065) cell lines, which both express GRP78 on their cell surface, were used in this experiment. The MDA-MB-486 (ATCC HTB-132) cell line was used as negative control. Approximately 15,000 cell/well were grown and fixed using 4% paraformaldehyde. After blocking, V80 nanobody (5 μg) was added to each well and incubated for 1 h at 37 °C. The wells were then rinsed and

**TABLE 1** *Titer of eluted phage after each round of panning*

| *Rounds of pannings* | *First round* | *Second round* | *Third round* | *Fourth round* |
|---|---|---|---|---|
| Titer of eluted phage | $5. \times 10^8$ | $4.2 \times 10^6$ | $3.7 \times 10^5$ | $2 \times 10^5$ |

HRP-conjugated anti-HA antibody was added. Detection was performed using TMB as described above.

### 2.8. Immunofluorescence

Approximately 30,000 cells of each A549, HepG2, and MDA-MB-486 cell lines were cultured on a culture slide and incubated at 37 °C with 5% $CO_2$ overnight. The culture medium was removed, and the cells were fixed with 200 μL of 4% paraformaldehyde. After blocking with BSA (5%), 200 μL of V80 nanobody (100 ng/μL) was added to each well and incubated at 4 °C overnight. After washing, the anti-HA antibody was added, and the slide was incubated for 60 Min at room temperature. The cells were incubated with FITC-conjugated anti-mouse IgG (1:250) for 2 H in the dark. For nuclear staining, propidium iodide was added to the wells. Imaging was performed by Leica TCS SP5II confocal microscope.

## 3. Results

### 3.1. Biopanning and screening

Selection of phage particle expressing anti-GRP78 nanobody was performed via four rounds of biopanning. After each round, phages were titrated then amplified and approximately $10^{13}$ phages were used for next round of panning. As shown in Table 1, the most eluted phage was obtained in the first round of panning.

For screening, 300 individual clones were randomly picked, and their binding affinity to CGRP evaluated by ELISA. More than 200 clones with low binding affinity to CGRP were excluded. From clones showing a good affinity toward CGRP, 20 clones with highest $OD_{450}$ were selected for sequencing (Fig. 1). One clone showing the highest affinity and specificity, named V80, was selected for further evaluation. Amino acid sequence including complementarity-determining regions (CDRs) and framework (FRs) of this nanobody is shown in Table 2.

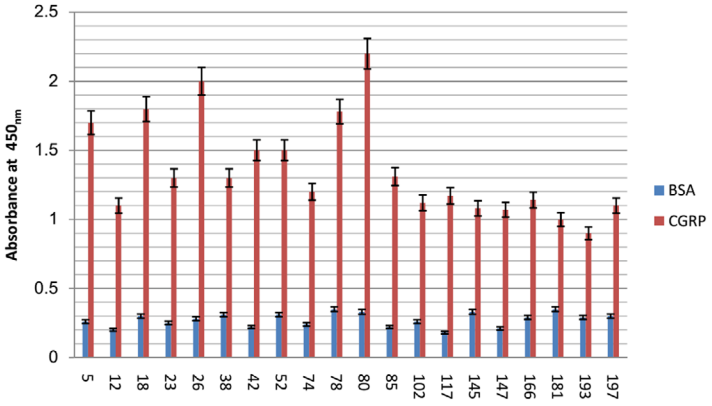


**FIG. 1** *ELISA results of 20 selected clones using CGRP as antigen.*

### 3.2. Cloning and soluble expression of nanobody (V80)

For soluble expression and characterization of V80, a DNA fragment encoding VHH was cloned in pET28a(+) vector and expressed in *E. coli*. Nanobody was expressed as a His-tagged recombinant protein and purified by Ni-NTA resin. A protein band of around 20 kDa responding to V80 appeared on polyacrylamide gel (Fig. 2). The purity of the recombinant protein was estimated to about 95% by the appearance of a single band on poly acrylamide gel.

### 3.3. Specificity evaluation of V80

For specificity determination, ELISA was performed on GRP78 and five irrelevant proteins as controls, using V80 as a primary antibody. As shown in Fig. 3, V80 nanobody specifically recognized GRP78 (OD > 2), and no cross-reactivity (OD < 0.5) was observed between V80 and other proteins used in this experiment. To evaluate binding potency of V80 to the GRP78 expressing cells, cell ELISA was performed. V80 nanobody could significantly bind to cancer cells expressing a high level of GRP78, whereas the interaction between negative GRP78 expressing MDA-MB-486 cell and V80 nanobody was very low (Fig. 4).

### 3.4. Affinity determination

The affinity of nanobody toward GRP78 was determined by SPR. Four different concentrations of nanobody interacted with GRP78. SPR sensor response overlay and Langmuir isotherm plot of the interactions are shown in Fig. 5. The calculated affinity of V80 was $2.1 \times 10^{-7}$ M.

**TABLE 2** *Amino acid sequence of anti-GRP78 VHH*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| V 80 | EVQLQQSGGGSVQAGGSLRLSCVAS | GYTRIHNH | VGWFRQDSGKEREGVAA | IYTGDGT | QTYAASVKGRFTISHDNANNTVYLQMNSLKPEGTGMYYCAQ | GAYSDIPLLIYDYDT | WGQGTLVTVSS |
| | FR1 | CDR1 | FR2 | CDR2 | FR3 | CDR3 | FR4 |

The CDRs sequence is indicated by red letters.

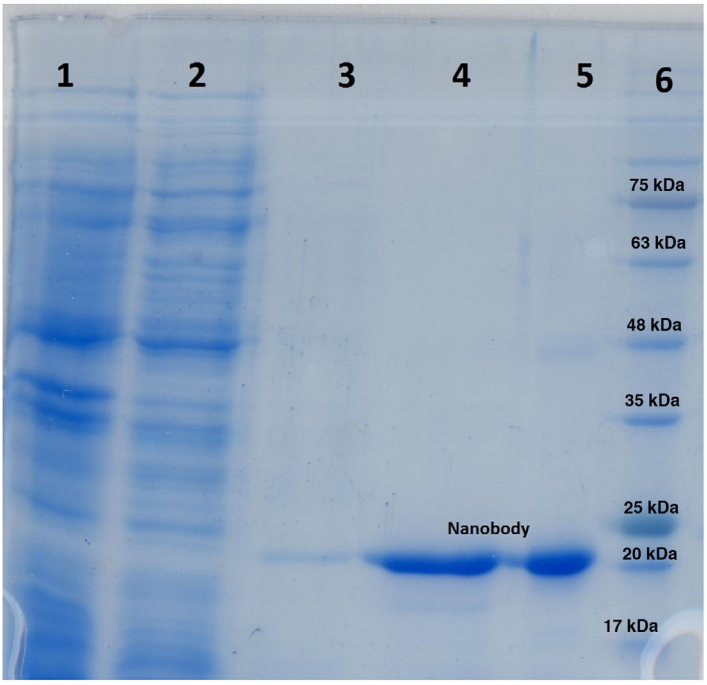


**FIG. 2** *SDS- PAGE analysis of V80; lane 1: uninduced* E. coli *soluble proteins. Lane 2:* E. coli *soluble proteins after expression induction with IPTG. Lanes 4 and 5: purified V80 nanobody by Ni-NTA resin. Lane 6: protein marker.*

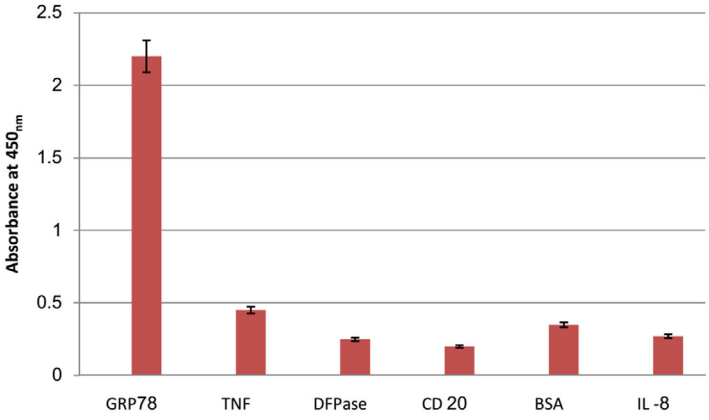


**FIG. 3** *Specificity assay for V80: purified nanobody showed interaction to GRP78. Interaction with irrelative proteins is in the baseline.*

### 3.5. Immunofluorescence and confocal microscopy of cancer cells

For confocal microscopy, cancer cells were first incubated with V80 nanobody and then stained with FITC-conjugated antibody. The appearance of green signals on the surface of A549 and HepG2 cell lines confirmed the presence of high amounts of GRP78 on their surface as well as their successful attachment to V80 nanobody. The absence of staining signals in the GRP78 negative cell line (MDA-MB-486) indicates the specificity of nanobody to GRP78 (Fig. 6).

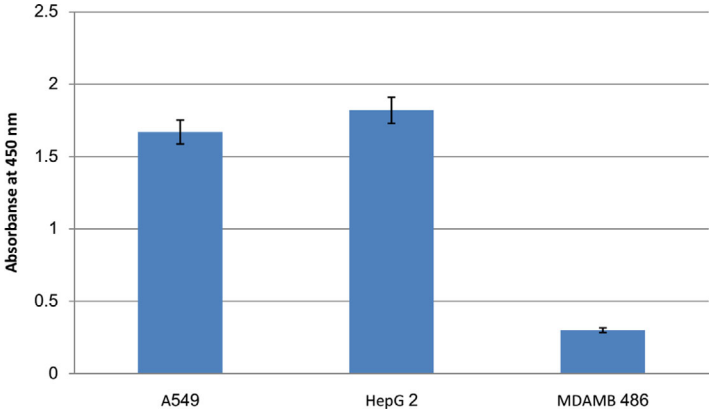


**FIG. 4** *OD measurement after Cell ELISA using V80 nanobody. Anti-GRP78 nanobody showed specific interaction with cell lines expressing GRP78 (A549 and HepG2.). MDA-MB-486 cell line (negative control) showed baseline signal in this assay.*

## 4. Discussion

Surface expression of GRP78 in cancer introduced it as a suitable target for cancer immunotherapy. Developing monoclonal antibodies directed against different domains of GRP78 is reported by different researchers. Misra et al. [17] showed that an antibody directed against the carboxyl domain of GRP78 led to induction of p53 and apoptosis in prostate and melanoma cell lines. A monoclonal IgG antibody named Mab 159 was reported to bind to cell surface GRP78 with high affinity ($K_d = 1.7$ nmol/L) and inhibiting tumor cell proliferation in breast carcinoma and colon cancer cell lines [29]. The mouse antibody directed against CGRP also induced apoptosis in melanoma cell and decreased cell proliferation in murine B16F1 melanoma flank tumor model [30]. A human IgM antibody isolated from a patient suffering from gastric cancer was able to bind to surface GRP78 and induce apoptosis in myeloma cells [31, 32]. However, conventional antibodies have some limitations when used for cancer detection and treatment. Large size (~160 kDa), low penetration in solid tumors, and high immunogenicity are some of these limitations.

Nanobodies are ideal probes in the diagnosis and molecular imaging due to their unique properties such as their small size, low immunogenicity, pH and temperature tolerance, high affinity to specific targets, high penetration to various body compartments, and fast clearance [33].

Phage display is a powerful technology for selection and generation of antigen-specific VHHs from libraries [34]. Since GRP78 is overexpressed and presented on the surface of cancer cells, we isolated a nanobody as a detection tool which specifically binds to GRP78 expressing cancer cells. The libraries used to isolate nanobodies were obtained from camels immunized

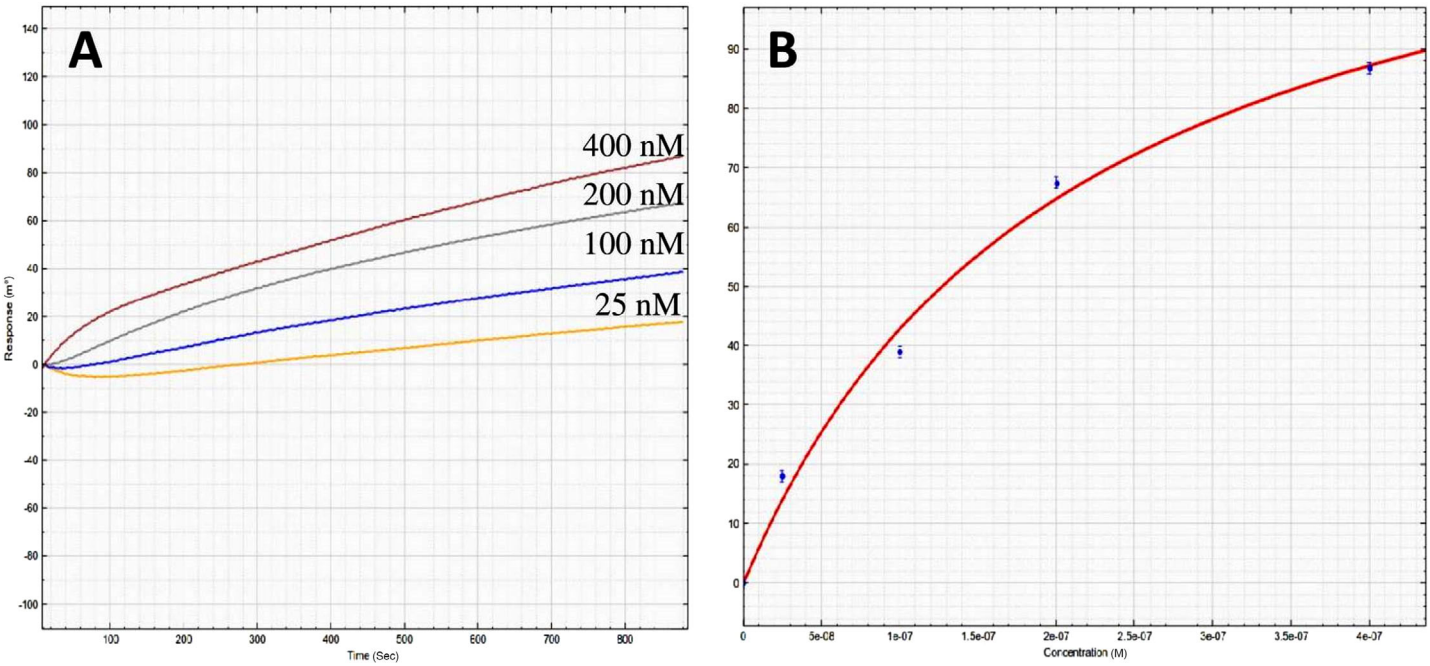


**FIG. 5** *SPR sensor response overlay plot for the interaction of different concentrations (25–400 nM) of nanobody with immobilized GRP78 (A). Langmuir isotherm plot of equilibrium angle (Req) versus nanobody concentration (B).*

with different cancer cells and antigens. Since GRP78 is overexpressed on most cancer cells, the isolation of nanobody binders to this protein from these libraries was suitable and shows to be an effective approach. For antigen binder selection, Pardon et al. proposed screening of 96–192 clones per target [27]. In the present study, we selected 300 colonies and evaluated their binding potency to CGRP. Further analyses to identify high-affinity clones led to the selection of V80 as a potent binder. Functional and soluble expression of V80 in *E. coli* confirmed our previous reports on this bacterium as a suitable host for recombinant production of nanobodies [23, 35-37].

In this study, colony selection was performed by ELISA using CGRP as the target antigen (Fig. 1). V80 showed high affinity toward CGRP with an $OD_{450}$ ratio of about 7.3 over nonspecific BSA. For specificity analysis, we used GRP78 as a positive control (Fig. 3). The result showed that V80 also had a high affinity to GRP78. Thus, V80 can bind to GRP78 and CGRP effectively. It can be concluded that the recombinant CGRP was folded in the correct manner and has a similar conformation to that of native GRP78. Structural similarity of recombinant CGRP to native GRP78 was predicted in our previous report [21]. The higher immunoreactivity of GRP78 expressing cancer cells (A549 and HepG2) compared with the GRP78 negative cell (MDA-MB-486) in cell ELISA, indicates the efficacy and specificity of our nanobody. Immunostaining of two cancer cell lines by V80 also exhibited the functionality of this antibody. The ability of this nanobody in binding to the

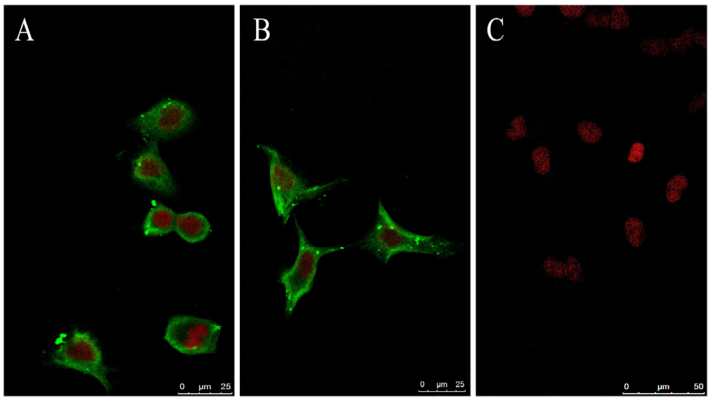


**FIG. 6** *Immunofluorescence of cancer cells using V80 nanobody: HepG2 (A) and A549 (B). Cell lines that expressing high GRP78 showed green signal. MDA-MB-486 (C) that used as a GRP78 negative cell line showed no staining signals on cell surface.*

surface of the cancer cell provides evidence of its potential application as a new tool for detection of cancer cells. However, further studies are needed to evaluate its utility in a clinical setting. Zhang et al. [38] reported the presence of multiple domains of GRP78, including CGRP, on the cancer cell surfaces. Presence of GRP78 on the surface of LoVo, SH-SY5Y, and A549 cancer cells was also demonstrated by immunofluorescence method [12]. A phage-displayed single-chain antibody isolated

by yeast two-hybrid screening also recognized GRP78 as its specific target. This human monoclonal antibody named Ab39 can bind to 45% of breast carcinomas, 35% of lung cancers, and 86% of melanomas showing a weak binding affinity to normal tissues in immunohistochemical analysis [15]. These findings are consistent with our results regarding the presence of CGRP on the cell surface.

For imaging of tumor tissues or induction of cell death, radioisotopes, including Indium-111, Gallium-68, copper-64, Lu-177, and 99mTC have been conjugated to nanobodies [39-42]. Since GRP78 is overexpressed on the cancer cells and V80 nanobody shows high specificity, its conjugation with such radioisotopes and further *in vitro* and *in vivo* assessments could make an ideal tool for the detection of cancer cells. To assess such application, we used FITC-conjugated antibody as a reporter and showed a specific signal for high GRP78 expressing cells while no signal was detected in GRP78 negative cells. Conjugating of nanobodies to fluorescent agents is reported in previous studies. The construct, called chromobody, was used for targeting antigens, specific intracellular compartment, measurement of enzymatic activity, identification of interacting factors, and manipulation of cellular function [43, 44].

As conclusion, we developed and introduced a new nanobody directed against GRP78. Using different strategies, we showed that this single chain nanobody could bind to the cell surface of different cancer cells, confirming the presence of CGRP on the surface of these cells. Application of this nanobody for inhibition of cancer cells growth can be studied by conjugation of V80 to cytotoxic agents such as bacterial toxins, nanoparticles, and radioisotopes. After confirmation by further experiments such as immunohistochemistry studies and Single Photon Emission Computed Tomography, V80 can be proposed as a novel tool to detect different cancer cells.

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The authors declare no conflict of interest.

## 6. Authors' Contribution

H.A. and S.L.M.G. studied the concept and design, drafted the manuscript, and made a critical revision of the paper. M.G. studied the concepts. M.R. studied the concept and contributed with the development of the protocol. L.A.M. worked for the development of the protocol. H.B. contributed with the development of the protocol and drafting the manuscript. M.F. analyzed the data. R.T. participated in the designing and performing some experiments for the study.

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