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## Isolation and characterization of nanobodies against epithelial cell adhesion molecule as novel theranostic agents for cancer therapy

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### Abstract

Epithelial cell adhesion molecule (EpCAM) plays an important role in tumorigenesis. Camelids produce functional antibodies composed of heavy chains only that bind to their antigens via a single domain variable fragment known as nanobody. Nanobodies show multiple advantages over traditional monoclonal antibodies. Isolation of functional anti-EpCAM nanobodies (Nbs) was the main aim of this study. An immune nanobody library containing  $10^8$  members was constructed previously. Anti -EpCAM nanobodies were isolated from camel immune library using phage display. Four consecutive rounds of biopanning were performed on immobilized EpCAM. Four nanobodies (Nb4, Nb5, Nb22, and Nb23) with highest signal intensity in monoclonal phage ELISA were selected. Affinity of these selected nanobodies for EpCAM was in the nanomolar range. Selected nanobodies significantly inhibited proliferation of MCF-7 cells. The *in vivo* study revealed that a significant reduction in tumor size occurred when treated with nanobodies Nb4 and Nb5, after 14 days monitoring. Our data revealed that nanobodies Nb4 and Nb5 could be considered as attractive theranostic agents for EpCAM overexpressing cancers.

**Keywords:** EpCAM, Single domain antibody, Phage display, Cancer, Theranostic agents

### 1 Introduction

Epithelial cell adhesion molecule (EpCAM) is an epithelial transmembrane glycoprotein encoded by TACSTD1 gene. EpCAM plays an important role in cancer proliferation, invasion, and metastasis [1]. It overexpresses in many cancers including breast, gastrointestinal, prostate, gallbladder, lung, and pancreas cancers [2, 3]. In recent decades, different cancer therapies have been developed such as radiation, chemotherapy, and surgery [4]. However, conventional

treatments suffer from off-targeting, insufficient drug concentrations in cancerous tissues, severe systematic toxicity, and even drug-resistance. For solving these issues, targeted therapy can be employed. The main approach in targeted therapy is the use of monoclonal antibodies to target the site of action [5]. European commission approved the first monoclonal antibody against EpCAM, called Catumaxomab, for malignant ascites [6]. In 2011, a bispecific T-cell-engaging (BiTE) antibody, named MT110, was approved for pancreas cancer, which targets simultaneously T-cell receptor CD3 and EpCAM. It was shown that MT110 exhibits an antitumor activity in diverse xenograft models [7]. However, monoclonal antibodies have a high specificity to the target, but low permeability to solid tumors, complex manufacturing process, and high cost of production restrict their applications in cancer therapy [8]. The antigen-binding fragment of homodimeric heavy chain-only antibodies are comprised in the single variable domain, known as VHH of Nanobody (Nb). VHHs are composed of four conserved regions encompassing three hypervariable complementarity-determining regions (CDRs) that involve in antigen recognition and binding [9]. VHHs are much smaller and have a higher tissue penetration rate than conventional antibodies, and possess superior properties including a high solubility, stability and resistance to heat-denaturation [10]. Therefore, a great deal of attention was paid to use them in cancer treatments both for diagnostic and therapeutic purposes.

This study describes the characterization of novel nanobodies against human EpCAM that were isolated from an immune nanobody library by phage display. Affinity of anti-EpCAM nanobodies were determined using ELISA. A competition assay was used to determine the binding capacity of selected nanobodies to the soluble antigen. *In vitro* biological activity was assessed on both overexpressing EpCAM (MCF-7) and non-expressing EpCAM (HEK 293) cells. Finally, the ability of tumor inhibition was investigated in a mice model.

## **2 Materials and Methods**

### **2.1 Reagents**

Anti-mouse HRP conjugated, and anti-M13-HRP antibodies were purchased from Roche, USA. All antigens used in the study were purchased from R&D Systems, Minneapolis, MN, USA. Anti-His HRP-conjugated antibody was obtained from Cytomatingen. VCSM13 helper phage was purchase from Amersham-Pharmacia. BSA, Casein, and Skim milk were from Merck. Maxisorp 96-well plate was purchased from Roskilde. Protein marker was purchased from Sinaclon, Iran. Immobilized-metal affinity chromatography resin was purchased from Qiagen. HUVEC (Human umbilical vein endothelial cells), MCF-7 (Michigan Cancer Foundation-7), and HEK 293 (Human embryonic kidney) cell lines were purchased from the cell bank of Pasteur Institute of Iran. The reagents for cell culture were purchased from Gibco. All other reagents used in this study were purchased from Sigma.

### **2.2 Biopanning**

Biopanning was performed to enrich nanobody-displaying phages against EpCAM. Nanobody-displaying phages were selected by four consecutive rounds of biopanning. The used nanobody phage-display library was prepared in our previous work [11]. Briefly, 200 µg of EpCAM antigen (expressed in HEK293 cells) and Freund's adjuvant (first injection was performed with complete Freund's adjuvant and subsequent injections were with incomplete Freund's adjuvant) was injected subcutaneously to a *Camelus Dromedaries* (young male) for six times at weekly interval. Before and after each injection, sera were collected and immune responses were

analyzed using ELISA. The next, camel blood sample was taken and peripheral blood mononuclear lymphocytes (PBMCs) were isolated. Total RNA was extracted, and cDNA was synthesized by reverse transcriptase. Then, VHH cDNA were amplified using nested PCR. Amplified VHH cDNA were digested with *Pst I* and *Not I* restriction enzymes and ligated into pHEN-4 phagemid which was digested with the same enzymes. Then, the recombinant phagemids were transformed into *E. coli* TG1 competent cells. Phage-display library containing  $10^8$  clones was rescued by infection of the primary library with  $10^{12}$  CFU of VCSM13 helper phage. At first, recombinant EpCAM protein was reconstituted in sterile sodium bicarbonate buffer (pH 8.4) at a final concentration of 10  $\mu$ g/ml. One microgram EpCAM was coated in each well of a 96 microtiter plate (Nunc, Roskilde, Denmark) and the plate was incubated overnight at 4 °C. Hundred microliter of sodium bicarbonate buffer was used as a control. The wells were blocked with 200  $\mu$ l blocking buffer (MPBS containing 4% (w/v) skim milk and PBS, pH 7.4) at room temperature (RT) for 1 h. The wells were then washed with PBS for three times. Then, phage particles ( $10^{12}$  CFU) were added to each well and incubated for 1 h at RT. The supernatant was then discarded, and the wells were washed 15 times with PBST 0.05 % (v/v) (PBS, containing 0.05% (v/v) Tween 20 adjusted to pH 7.4). The phages were eluted by adding 100  $\mu$ L triethylamine solution (100 mM TEA, adjusted to pH 12.0) to the wells and the plate was incubated at RT for 10 min. Phage-containing supernatants were transferred to a new tube and immediately neutralized using 1M Tris-HCL (pH 8.0). Exponentially growing *E. coli* TG1 cells (OD<sub>600nm</sub> 0.4-0.6) were infected with 10-fold serially diluted phages and each phage titration was cultured on TYE medium containing ampicillin (100  $\mu$ g/mL). The eluted phages were then used to infect 5 ml of freshly prepared *E. coli* TG1 cells for phage amplification. The amplified phages were rescued with VCSM13 helper phage. To purify the phages, the bacteria were centrifuged at 5000  $\times$ g for 15 min, and recombinant phages in the supernatant resuspended in PEG/NaCl solution (20% w/v PEG 6000, 2.5 M NaCl), and incubated on ice for 1 h. The phage particles were finally precipitated by centrifugation at 10000  $\times$ g for 20 min, resuspended in 1 ml PBS, and stored at -20 °C. The stringency of biopanning was increased through increased concentration of tween 20 in PBST buffer (0.05, 0.1, 0.15 and 0.2 %) for subsequent rounds of biopanning[12-14].

### 2.3 Polyclonal phage ELISA

Polyclonal ELISA was used to verify the biopanning process after four rounds of biopanning. The ELISA microplate was coated with EpCAM antigen in PBS (0.5  $\mu$ g/well) and PBS was used as control. After washing with PBST 0.05 % (v/v) (PBS containing 0.1 % (v/v) Tween 20 ), all wells were blocked with 200  $\mu$ l blocking buffer (4% (w/v) skim milk) and incubated at RT for 1 h. The wells were then washed 15 times. The phage solution over four rounds of panning ( $\sim 10^9$  CFU) was added to each well and incubated at RT for 1 h. After extensive washing, the anti-M13-HRP conjugated antibody was added (1:5000 dilution) and then plate incubated at RT for 1 h. After washing, 100  $\mu$ l TMB (3, 3', 5, 5'-Tetramethylbenzidine) was added to each well and the plate was incubated for 10 min at RT. After incubation, the reaction was stopped by adding 100  $\mu$ l of 2N sulfuric acid. Finally, optical density was measured at a wavelength of 450 nm using a UV/Vis spectrophotometer (Epoch, BioTek, USA).

## 2.4 Phage monoclonal ELISA

Over 112 clones were randomly picked from 3<sup>rd</sup> and 4<sup>th</sup> rounds of biopanning. In brief, EpCAM antigen (1 µg/mL in PBS) was coated in each well of 96-well plates overnight at 4 °C. Ten µg/ml of BSA in sodium bicarbonate buffer was used as negative control. Afterward, the plates were blocked using PBS supplemented with 0.3% skim milk for 1 h at RT. After washing with PBST 0.05% , 10<sup>12</sup> CFU of eluted phages were added to the wells and the plates were incubated at RT for 1h. After washing, the plates were incubated with an anti-M13-HRP monoclonal antibody (1:2000) at RT for 1h. Hundred microliter of TMB was then added and the plate was incubated for 10 min at RT. The reaction was finally stopped by adding 100 µl of 2N sulfuric acid. Colony PCR was performed on multiple binding phages with a high signal at a wavelength of 450 nm. The binding phages were subjected to the sequencing and the sequences were analyzed in NCBI-BLAST.

## 2.5 Cloning, expression, and purification of selected Nanobodies

The selected nanobody coding sequences were amplified using forward (5-GATGTGCAGCTGCAGGAGTCTGGGGGAGG-3) and reverse primers (5-GGACTAGTGC GGCCGCTGGAGACG-GTGACCTGGGT-3). The coding sequences were sub-cloned into pHEN6c expression vector using *Pst*I and *Bst*EII restriction enzymes. PelB and 6X-His sequences were added to the N-terminus and C-terminus of construct, respectively. The recombinant constructs (pHEN6c-Nb) were transformed into competent *E. coli* WK6 cells prepared by the heat-shock method. Expression of nanobodies were induced with 1 mM IPTG and overnight incubation in a shaker incubator at 28 °C. The cultures (300 ml) were centrifuged at 5000 xg for 10 min and the periplasmic proteins were released using TES (30 mM Tris-HCl, 60 µl 0.5M EDTA, 20% sucrose pH 8). About 5 mg of bacterial pellet was resuspended in 12 ml of TES buffer. The His-tagged nanobodies were purified by an immobilized metal affinity chromatography (IMAC, QIAGEN, Germany). The proteins were eluted with PBS containing 250mM imidazole, dialyzed twice against PBS at 4 °C, and concentrated using a amicon filter (cut off 5 kDa). Then, gel filtration chromatography superdex-75 was performed on eluted nanobodies (GE Healthcare Life Sciences, USA). The expression of nanobodies was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE) and coomassie brilliant blue staining method. In western blotting, the protein bands were transferred to a nitrocellulose membrane. The membrane was blocked with a solution containing 4% skim milk for 16 h at 4 °C and then washed with PBST. The identification was performed using a HRP-conjugated anti-His antibody (1:2000 in PBS) and the bands were developed by DAB substrate[15]. The protein concentration was measured using the UV spectrophotometer absorption at 280 nm and extinction coefficient was used for calculation.

## 2.6 Binding analysis

The binding analysis of nanobodies was examined using ELISA. At first, different antigens including Neuropilin-1 (NRP-1), programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PDL-1), zinc transport protein LIV-1, and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) , human epidermal growth factor receptor 2 (HER-2) were coated into 96-microplates at a final concentration of 36 nM. BSA 1 % and skim milk 4 % was used as control. After blocking and washing steps, the binding was evaluated by adding anti-His HRP-conjugated antibody (1: 2000 in PBS)[16].

## 2.7 Affinity

The affinity of expressed nanobodies to the antigen was determined by modified Beatty *et al.* method [17]. In short, microtiter plate was coated with 100  $\mu$ l of two different concentrations of EpCAM antigen (1 and 10  $\mu$ g/ml) and BSA as control and incubated at 4 °C overnight. Then, the plate was blocked at RT for 1 h. After washing with PBST 0.05 %, serial dilution of nanobodies (0–10 nM) were added to the wells and the plate was incubated for 1 h at RT. After washing, HRP-conjugated anti-His antibody (1:2000) was added to each well. Finally, the peroxidase activity was measured by adding TMB at a wavelength of 450 nm using a UV/Vis spectrophotometer (Epoch, BioTek, USA). The affinity was then calculated using the following formula:

$$K_{\text{aff}} = 1/18 (20[\text{Nb}]_t - 2[\text{Nb}]_t)$$

## 2.8 Competitive ELISA assay

A competition assay was used to determine the binding capacity of selected nanobodies to the soluble antigen. EpCAM antigen and BSA (1  $\mu$ g/ml) were coated in 96-well microplate at 4 °C overnight. After that, different concentrations of nanobodies were individually mixed with soluble EpCAM for 1 h at 37 °C and then added to the coated wells. The percentage of bonded nanobodies was determined by a home-made ELISA. Briefly, HRP-conjugated anti-His antibody was mixed for 1 h at RT followed by adding HRP substrate. The concentration of soluble EpCAM that prevents binding of soluble nanobody to the immobilized antigens, was measured at 450 nm.

## 2.9 MTT assay

The HEK 293 and MCF-7 cells ( $4 \times 10^4$  cells/well) were cultured in 96-well plates containing DMEM supplemented with 10% FBS and 1X PenStrep in a 5% CO<sub>2</sub> incubator. HUVEC cells ( $4 \times 10^4$  cells/well) were cultured in a 96-well plate containing HAMS-F12 supplemented with 10 % FBS and 1X PenStrep in a 5% CO<sub>2</sub> incubator. The cells were then treated with increasing concentrations of anti-EpCAM nanobodies or anti-VEGF nanobody (irrelevant nanobody, internal control) (0.4-40 nM) and incubated for 24 and 48 h. After that, 10  $\mu$ l of MTT solution (5 mg/ml) was added to each well and the plates were incubated for 4 h at 37 °C in dark. The formazan crystals were solubilized by adding 100  $\mu$ L dimethylsulfoxide (DMSO). Finally, the absorbance was measured at a wavelength of 570 nm using a UV/Vis spectrophotometer (Epoch, BioTek, USA). The relative viability was calculated by the following formula [18-20].

$$\text{Relative Viability (\%)} = (\text{Abs}_{\text{treatment}} - \text{Abs}_{\text{blank}}) / (\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}) \times 100$$

Where Abs is of the measured absorbance for each sample.

## 2.10 Animal model for the analysis of antitumor efficacy

Nude mice (19-23 g, n = 4 mice/group) were obtained from the laboratory of Tehran University. The animal study was approved by the committee on the ethics of animal experiments of Pasteur Institute of Iran. Solid tumors were induced in mice by subcutaneous injection of  $2 \times 10^6$  HCT116 cells into the dorsolateral flank. The mice were then divided into six groups: Nb4, Nb5, Nb22, Nb23, control nanobody, and PBS. The mice were received nanobodies through the vein tail by an intravenous (i.v.) injection at a dose of 10 mg/kg twice a week for four weeks. The

tumor size was monitored twice a week using a caliper by the following formula for 9 days. After 9 days, the tumor was removed and weighted[16, 21].

$$\text{Tumor Volume} = 0.5 \times \text{length} \times \text{width}^2$$

## 2.11 Statistical analysis

GraphPad Prism 7 was used to perform regressions and curve fittings (GraphPad Software, Inc., La Jolla, CA, USA). The significance level between the two groups was analyzed using a two-tailed unpaired *t*-test. The significance level between more than two groups was investigated by one-way or two-way analysis of variance. A *P*-value of less than 0.05 was considered as a statistically significant difference between the groups.

## 3 Results

### 3.1 Library enrichment

Biopanning was performed to isolate anti-EpCAM nanobodies. Nanobodies were enriched by four rounds of biopanning. The phage particle ratio significantly increased after the 3<sup>rd</sup> round. Successfully, increasing of phage titers was observed after three and four rounds of biopanning compared with the negative control. Polyclonal phage ELISA confirmed the specific binding of the eluted phages in each round that indicates success of the selection process (**Table 1**). The 3<sup>rd</sup> and 4<sup>th</sup> rounds were showed the highest signals in the polyclonal phage ELISA (**Figure 1**). Over 112 individual clones were chosen from the 3<sup>rd</sup> and 4<sup>th</sup> rounds of biopanning and were subjected to a monoclonal phage ELISA. The results showed that ten clones reacted specifically with EpCAM (**Figure 2**). The selected clones were then sequenced and multiple-aligned using NCBI BLAST service (**Table 2**).

< Figure 1 >

< Table 1 >

< Figure 2 >

< Table 2 >

### 3.2 Expression and purification of anti-EpCAM nanobodies

The selected nanobodies were expressed, purified, and analyzed on a 12% SDS-PAGE electrophoresis gel (**Figure 3A**). A 17 kDa band was observed for all expressed nanobodies, and the production yields were ~40-70 mg/L. Isolated nanobodies were identified by western blot analysis using an HRP-conjugated anti-His antibody (**Figure 3B**).

< Figure 3 >

### 3.3 Binding capacity of anti-EpCAM nanobodies

Binding capacity of nanobodies was investigated by ELISA. The results showed that all nanobodies detected EpCAM in ELISA experiment and no cross-reactivity was observed with other antigens (**Table 3**).

< Table 3 >

### 3.4 Affinity of anti-EpCAM nanobodies

Affinity of nanobodies to EpCAM was calculated according to the Beatty method. The calculated affinities ( $k_{\text{aff}}$ ) were  $5.2 \times 10^{10}$ ,  $3.8 \times 10^{10}$ ,  $1.5 \times 10^{10}$ , and  $5 \times 10^9$   $\text{M}^{-1}$  for Nb4, Nb5, Nb22, and Nb23, respectively (**Figure 1S and Table 1S in supplementary data file**).

### 3.5 Competition ELISA assay

A competition ELISA assay was used to check the detection of EpCAM soluble form by the selected nanobodies. The results showed that all four nanobodies could detect EpCAM in the solution phase (**Figure 4**).

< Figure 4 >

### 3.6 MTT assay

The cytotoxicity of anti-EpCAM nanobodies was assessed by a MTT assay on MCF-7 and HEK293, and HUVEC cell line. The results indicated that, even after 48h, no cytotoxicity was observed in HUVECs due to a lack of EpCAM receptor in the cell surface (**Figure 2S in supplementary data file**). In MCF-7 cells, a high decrease in the cell viability ( $p < 0.05$ ) was observed after 24 or 48h in a dose-dependent manner. Nb4 and Nb5 were the best nanobodies that could inhibit MCF7 cell proliferation. Anti-EpCAM nanobodies just moderately showed a dose-dependent cytotoxicity on HEK cells because of the low-level expression of EpCAM receptor on the cell surface (**Figure 5**). Control nanobody showed no toxicity on proliferation of the cells. The calculated  $\text{IC}_{50}$  values for MCF-7 and HEK293 cells were summarized in **Table 4**.

< Figure 5 >

< Table 4 >

### 3.7 In vivo tumor targeting

Tumorized nude mice were received a dose of 10 mg/kg from each nanobody twice a week for four weeks. After injections, size, as well as the weight of tumors were monitored for 9 days. The results indicated that all nanobodies significantly decrease the size and weight of tumors compared to the PBS or control nanobody received group (**Figure 6**).

< Figure 6 >

## 4 Discussion

Recent investigations revealed that EpCAM plays an essential role in cell-cell adhesion, signaling, cell migration, and differentiation. It also overexpresses in tumor and metastatic tissues and helps the cancer cells to spread out through lymphatic and blood vessels. Therefore, it is considered as an important tumor target for diagnostic and therapeutic purposes. Edrecolomab is the first mouse monoclonal antibody against EpCAM that specifically targets the antigen in the human gastrointestinal malignancies [22]. In preliminary studies, Edrecolomab did not show a consistent clinical benefit in the advanced stages. However, it well tolerated in many terminal malignant patients without significant toxicities [23, 24]. The next monoclonal antibody,



Adecatumumab, had a moderate affinity for EpCAM and showed efficacy in clinical trial studies for metastatic breast cancer. However, in earlier clinical trials, it was associated with some toxic effects [25, 26]. Although such antibody-based strategies benefit from high specificity and high-affinity of monoclonal antibodies to target the cancer cells, but they are usually associated with some pharmaceutical, pharmacological, and pharmacokinetic issues compared to the small molecules [8]. For example, the large size (approximately 150 kDa) often decreases the penetration rate of monoclonal antibodies into solid tumors. Superior advantage of nanobodies over conventional antibodies includes excellent tissue penetration[27-29]. These characteristics make them an ideal tool for tumor targeting approaches[30]. One of the best ways for isolating specific nanobodies from the camelid libraries is biopanning [31]. For the first time, anti-EpCAM nanobodies were isolated and characterized from an immune nanobody library using the phage-display technique. In the current study, four rounds of biopanning were carried out and the selected nanobodies were characterized using further assays. With an increase in the stringency of the washing steps in each round of panning, the affinity of the binders was increased. A polyclonal phage ELISA was used to determine the best round of panning. The results indicated that the phages from rounds 3 and 4 had good binding capacity to immobilized EpCAM. Finally, four clones that showed strong signals in monoclonal phage ELISA were chosen for further characterizations [31]. Colony PCR showed the proportional size of nanobody as insert. Results of sequencing showed that each clone contained a different CDR3 sequence. All four nanobodies were successfully purified using affinity chromatography in large yields (~0.04–0.07 g/L) which is almost equal to the expression of NbBruc nanobodies[32]. Our results showed that the binding intensity of soluble nanobodies were significantly higher than that of the nanobodies expressed on the surface of phages. This increase may be explained by better interaction between the soluble nanobodies and the coated antigen. To investigate the binding capacity of selected nanobodies, various antigens were examined for cross-reactivity measurements.

The Beatty method was used to determine the affinity of selected nanobodies. The affinity of selected nanobodies was in nanomolar range similar to currently available anti-EpCAM monoclonal antibodies that are suitable for targeting purposes. However, Adecatumumab is a fully human IgG1 antibody with affinity of  $10^{-8}$  M [33-35]. Edrecolomab is a murine monoclonal antibody to Ep-CAM with low-affinity and limited antitumor efficacy[36-41]. Different affinities obtained for each nanobody indicated that they might recognize different epitopes in the antigen. Competition ELISA was used to determine the detection capability of nanobodies in insoluble and soluble phases. Coated antigens immobilized at different conformations. Therefore, it is important to check the ability of nanobodies to detect the antigen in the soluble form that is more likely to the native conformation. In a similar study, in competition immunoassay, antibodies were attached to the soluble antigen and prevented the attachment of antibodies to the insoluble antigen [42].

The MTT assay results revealed that the nanobodies successfully inhibit the proliferation of MCF-7 cells (expressing EpCAM). This inhibition is well correlated to the level of receptor expression in the studied cell. Since EpCAM is overexpressed in breast cancer and responsible for proliferation and distribution of neoplasm [43-45], and is considered as a potential target in breast cancer. Therefore, it is necessary to select appropriate antibodies against EpCAM and used as drug for cancer therapy. [46]. In addition, animal study demonstrated that all of nanobodies inhibited the tumor growth *in vivo*.

## 5 Conclusion

In conclusion, anti-EpCAM nanobodies were isolated and characterized using phage display method. The results showed that high affinity anti-EpCAM nanobodies were selected. Selected nanobodies exhibited a different range of toxicity to EpCAM expressed tumor cell lines *in vitro*. Selected nanobodies inhibited the tumor growth in a mice model. Our results indicate for potential of anti-EpCAM nanobodies as promising therapeutic agent for cancer-targeted therapy.

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## Conflict of interest

The authors declare there is no conflict of interest.

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## Tables

**Table 1.** Enrichment results of phage particle after biopanning on EpCAM antigen.

Round	Number of binding phages (CFU)	Number of non-binding phages (CFU)	Enrichment ratio (Bp/Np)*
1	200×10 <sup>4</sup>	100×10 <sup>4</sup>	2
2	214×10 <sup>4</sup>	104×10 <sup>4</sup>	2.11
3	168×10 <sup>5</sup>	29×10 <sup>4</sup>	57.9
4	75×10 <sup>6</sup>	23×10 <sup>4</sup>	326

\*Bp: Number of binding phages

\*Np: Number of non-binding phages

**Table 2.** The amino acid sequences of selected nanobodies.

Nbs	FR1	CDR1	FR2	CDR2
Nb 4	QVQLVQSGGGSVQGGASLRLSCAAS	GGERNNYCVA	WFRQAPGKEREVAA	ISRAASGAQTTTKYYVD
Nb 5	QVQLVQSGGGLVQGGASLRLSCAAS	SGRAGLIHVA	WFRAKNTLYRQVAA	ISSDDTG FAT-LYYAVD
Nb 22	QVQVQSGGGSVQAGASLRLSCAAS	SGLQQAIYVA	WFRQAVGKEREVAA	IGYWYWHIQVYKYYVD
Nb 23	QVQLVQSGGGSVQAGASLRLSCAAS	SKHRQLILVA	WFRQASGKEREVAA	ISYWYWHIITAGKYVD

Nbs	FR3	CDR3	FR4
Nb4	SVKGRFTISQDTKNTATVYLQMNSHKPEDTAYCT	AKAKIYPPQCTGISRTIDY	RGQGTQVTVSS
Nb5	SVKGRFTISQDTESTKYSYLQMNSLKPEDTIYCT	AKLFSKTSYQKFIYkFNY	WGQGTQVTVSS
Nb22	SVKDRFTISGDTKST-KTYLQMNSLKNEDTAYYC	AKIHHLPRQRQEHGIFDY	RGQGTQVTVSS
Nb23	SVKGRFTISQDTKSTATLYNQMSLKPEDTAYCT	DKAKIYPPQCTGISRTFVY	RGQGTQVTVSS

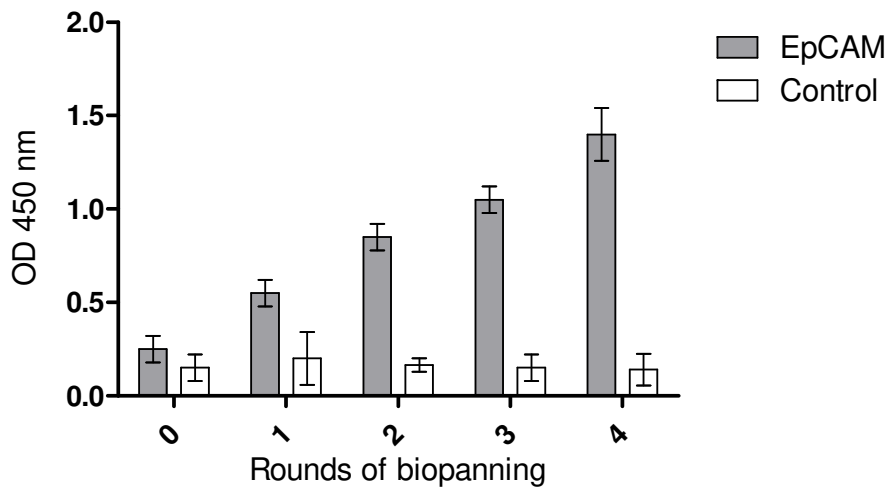
**Table 3.** The Binding analysis of nanobodies. The percentage of binding of nanobodies to different antigens are represented. The data are mean of three independent measurements±SD

Nanobody	EpCAM	PD1	NRP1	HER-2	BSA	Skim milk	CTLA_4	LIV_1	PDL1
Nb 22	52±7	13.4±3	18.4±3	4.2±2	3.2±2	3.8±2	14.6±3	18.8±4	15.3±2
Nb 4	90±5	15±3	21.2±2	10±4	4.2±5	4.6±2	15.3±4	11.2±1	17.3±2
Nb 23	50±4	15.7±5	14.2±3	5±3	3.8±4	3.2±3	12.3±1	10.4±3	10.8±2
Nb 5	96.2±4	17.3±3	16.1±4	4.3±3	5.7±2	3.8±1	16±2	8.5±3	13.7±3

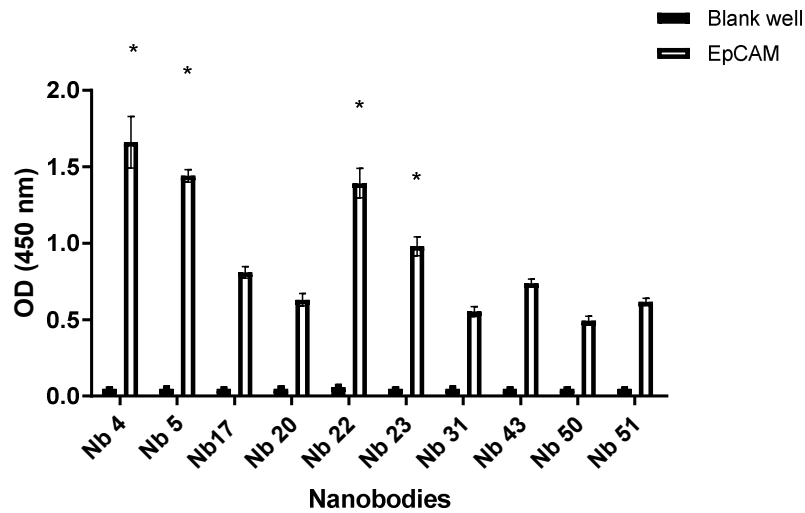
**Table 4.** The calculated IC<sub>50</sub> values (nM) for MCF-7 and HEK 293 cells at different time-points.

Time-points (h)	HEK 293		MCF-7	
	24	48	24	48
Nb 4	45	30	9	4
Nb 5	65	50	22	19
Nb 22	75	55	37	25
Nb 23	72	60	41	30

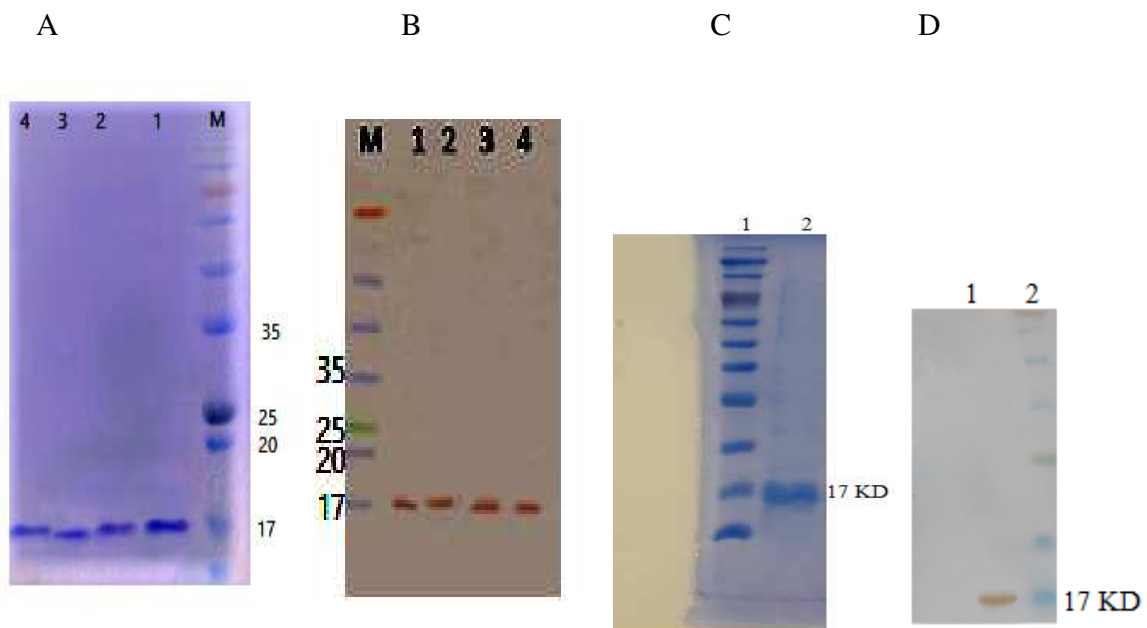
## Figures



**Figure 1.** Polyclonal phage-ELISA. As depicted in the figure, the 3<sup>rd</sup> and 4<sup>th</sup> rounds of biopanning were showed the highest absorbance. Data are represented as Mean  $\pm$  SD from three independent measurements.

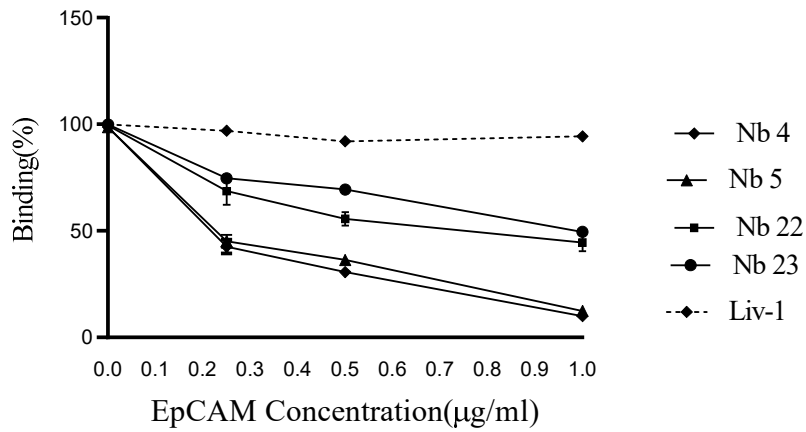


**Figure 2.** Monoclonal phage ELISA. Clones which indicated by asterisks were considered as binding phages. Data are represented as Mean  $\pm$  SD from three independent measurements.

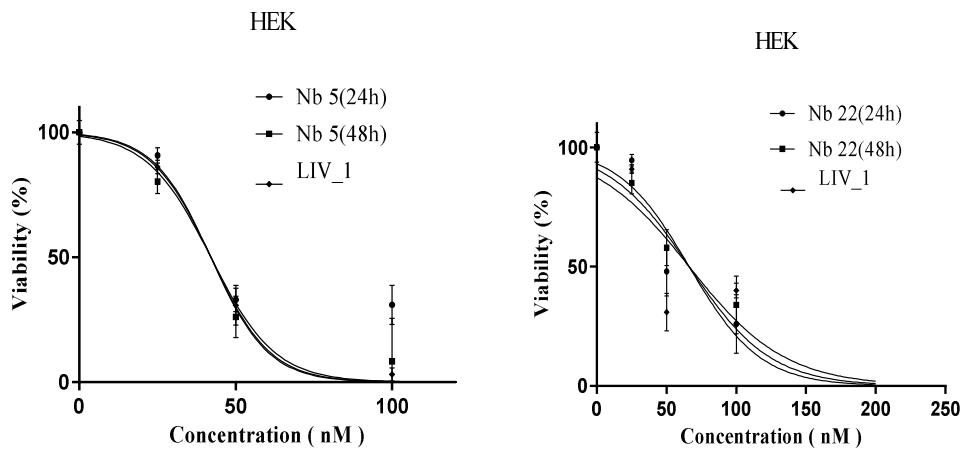


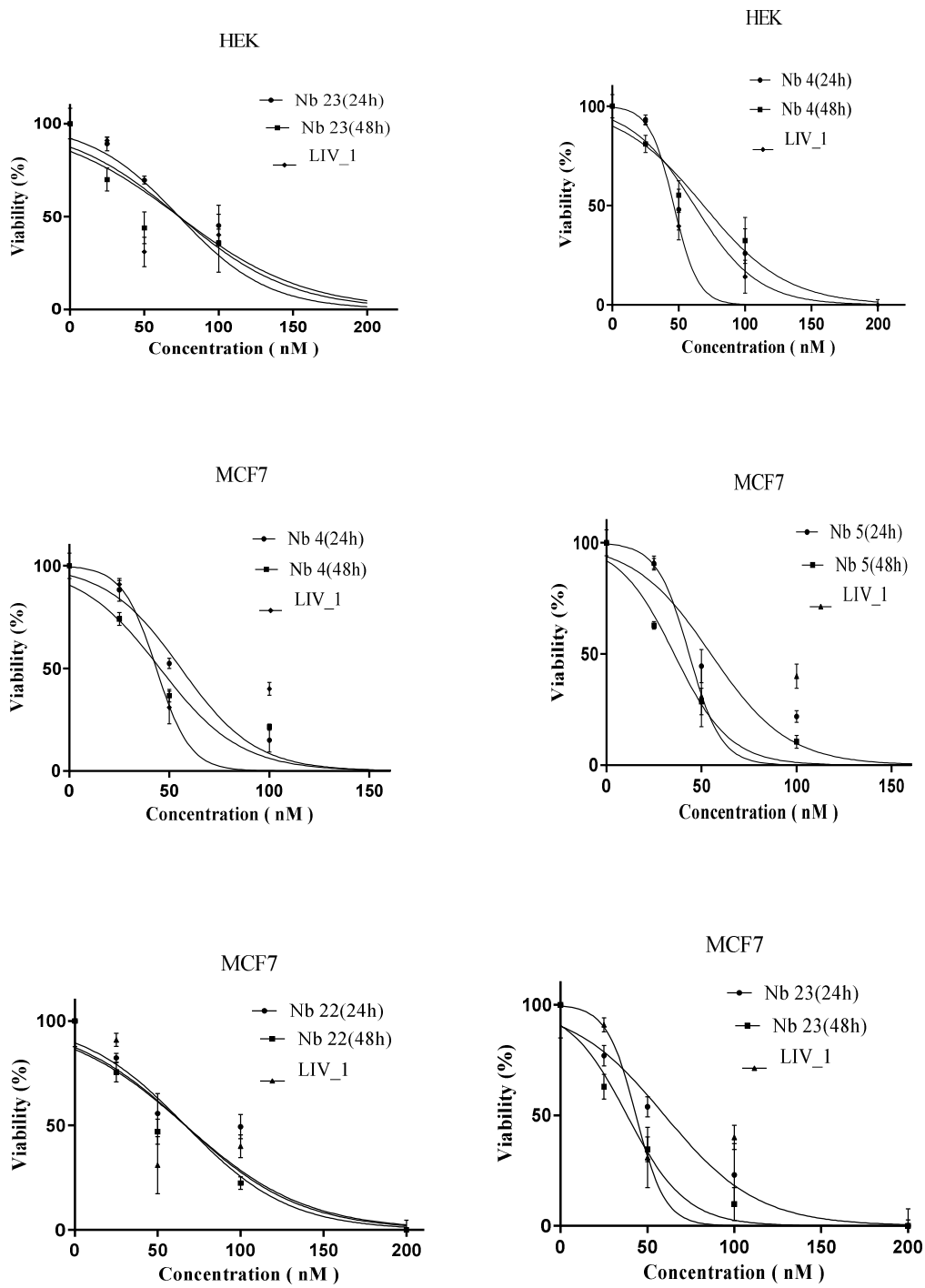
**Figure 3.** Purification results. (A) 12% SDS-PAGE and (B) Western blot analysis of purified nanobodies [M: molecular weight marker (kDa), lane 1: Nb4, lane 2: Nb5, lane 3: Nb22, and lane 4: Nb23].(C, D) anti-Liv-1 Nanobody as control nanobody



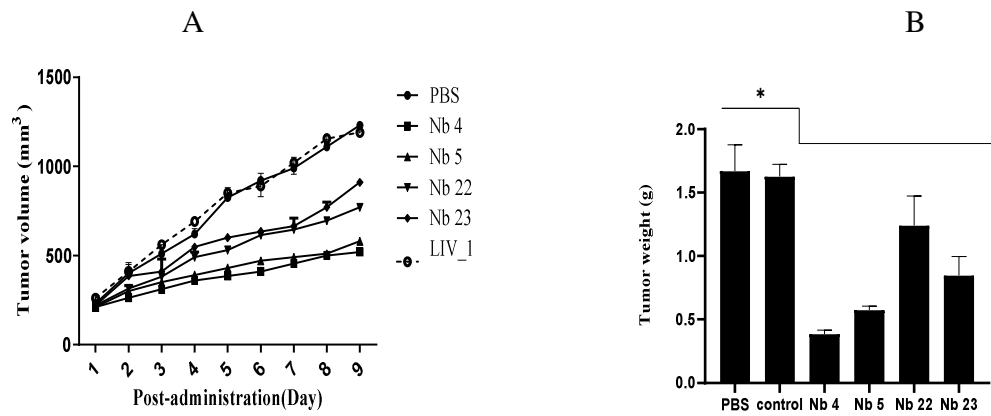


**Figure 4.** Competition ELISA assay. As depicted in the curve, nanobodies detected EpCAM in the solution phase. Anti-Liv-1 nanobody was used as control. The data are represented as mean  $\pm$  SD from three independent measurements.





**Figure 5.** Cell proliferation assay. The selected nanobodies showed cytotoxicity on MCF-7 and HEK293 cells in a dose-dependent manner. Nanobody against Liv-1 was used as control in all experiments. Data are expressed as mean  $\pm$  SD from three independent measurements.



**Figure 6.** *In vivo* antitumor activity of selected nanobodies. As depicted, all nanobodies inhibited both the (A) volume and (B) weight of induced tumors. Anti-Liv-1 nanobody was used as control. Data are represented as mean  $\pm$  SD from four independent measurements ( $*P < 0.05$ ).