Original Research Article

Utilization of Nanobodies in Immunoturbidimetric Assays

ABSTRACT

Aims: Nanobodies, characterized by their small size, accessibility, and high yield, are emerging as advantageous tools in various applications. Despite these benefits, their integration into the formulation of immunoturbidimetric reagents has remained limited since their discovery.
Methodology: This study utilized nanobodies specific to cystatin C to formulate immunoturbidimetric reagents and compared them with traditional rabbit polyclonal antibody reagents.
Results: The findings revealed that the multiantibody affinity of Cys-rPAB surpasses that of Cys-VHH-104 and Cys-VHH-704 nanobodies, although the difference is not statistically significant. Upon evaluating the reagent formulation performance, both types of antibodies were found to satisfy the prerequisites of conventional immunoturbidimetric assay reagents. The correlation coefficient R2 in the statistical analysis of the clinical results was 0.9986, signifying a high degree of consistency.
Conclusion: These findings underscore the feasibility and considerable economic potential of employing nanobodies in immunoturbidimetric reagents.

Keywords: Nanobodies; Immunoassay; Affinity; Cystatin C

1. INTRODUCTION

A specialized class of antibodies, referred to as nanobodies, has been sequentially discovered in two species, namely, camels and sharks. Professor Hamers-Casterman of the Free University of Brussels made the discovery in 1989 that antibodies derived from camels infected with Trypanosoma evansi were devoid of light chains. These antibodies, which lack light chains and are known as heavy-chain antibodies, are found within camelid species and are distinguished by the absence of the CH1 domain required for light chain pairing [1]. As a result, they contain only two heavy chains, with each chain encompassing a variable heavy chain domain (VHH) structural domain [2]. In 1998, Professor K.H. Roux uncovered novel immunoglobulin new antigen receptors (IgNAR) in nurse sharks, where the binding to antigens necessitates two independent, highly soluble new antigen receptors, of which the variable new antigen receptors (VNAR) is the smallest naturally occurring antibody-derived module, each with a molecular weight of approximately 12 kD [3]. With the progress of technology, researchers have elucidated the crystal structure of VHH/VNAR domains, with the VHH domain measuring approximately 2.5×4.0 nm and having a molecular weight of 15 kD (VNAR is 12 kD), as well as demonstrating the viability of selective identification, assessment, and recombinant expression of Nb. This has contributed to an expanding application of nanobodies in various research fields [4,5].

In comparison to conventional monoclonal antibodies (mAb), nanobodies (Nbs) exhibit unique structural benefits. Their diminutive size, pronounced shape, and extensive complementarity-determining region 3 afford them specific complementary sites, allowing binding to certain obstructed antigenic recesses that are typically inaccessible to larger conventional monoclonal antibodies [6]. In the realm of disease diagnosis, Nbs have been widely employed in double-antibody sandwich ELISA methodologies to identify biomarker proteins, viruses, or other pathogens [7].

Immunoturbidimetry represents an immunological detection methodology deployed in precipitation reactions by synergizing contemporary optical measurement instruments with automated analytical systems. This technique enables the quantitative detection of a multitude of trace antigens, antibodies, drugs, and other small molecular semiantigens within bodily fluids [8]. Cystatin C, a 13 kD cysteine protease inhibitor protein, is synthesized at a constant rate by all nucleated cells, is freely filtered by the kidneys, and undergoes almost complete reabsorption and metabolic degradation within the proximal tubules, with negligible urinary excretion [9].

The concentration of this substance remains stable and uninfluenced by variables such as age, sex, muscle mass, the majority of medications, and inflammation [10]. Consequently, cystatin C can function as a renal performance metric and is poised to become a biomarker for glomerular filtration [11]. Since its inaugural detection via turbidimetry in 1994, cystatin C has evolved into an emblematic product in biochemical immunology, extending from ordinary turbidimetry to latex-enhanced turbidimetry [12].

In clinical practice, Cys-C testing reagents are largely reliant on rabbit polyclonal antibodies and have exhibited satisfactory performance, elevating the testing level from g/L to mg/L, with some latex turbidimetric reagents even reaching μg/L detection levels [12]. Nevertheless, given the utilization of Ig-class antibodies (inclusive of polyclonals and monoclonals), challenges such as elevated cost, interbatch variations (in the case of polyclonals), and interference from heterophilic antibodies persist, leading to suboptimal accuracy and occasional false positives in clinical assessments. The theoretical application of recombinant nanobodies may offer solutions to these issues.

At present, the market lacks immunoturbidimetric reagents crafted with Nb antibodies. Within the context of this study, we isolated two strains of nanobodies from a previously established variable region library created with shark and camel nanobodies using the Cys-C antigen and conducted recombinant expression. We subsequently contrasted these recombinant Nbs with rabbit polyclonal antibodies concerning antigen affinity and various performance indicators, including calibration, clinical correlation, and precision, when formulated as Cys-C immunoturbidimetric assay kits. This investigation assesses the prospective application of Nb in in vitro diagnostic reagents and lays a scientific foundation for the innovation of new Cys-C immunoturbidimetric testing kits.

2. MATERIAL AND METHODS

2.1 Materials

The Cys-VHH104 nanobody, Cys-VHH704 nanobody, Cys-rPAB antibody, and Cys-C antigen were synthesized and prepared in our laboratory. JSR Corporation, Japan, supplied 10% 120 nm polystyrene microspheres. The 1x PBS was obtained from Pall Fortebio Company. Biotin at a concentration of 5 mg/ml was procured from Thermo Company. Additionally, disposable PD-10 Sephadex G-25 M columns and Greiner 96-well black microplates were furnished by Pall Fortebio Company. Beijing Kangwei Century Biotechnology Co., Ltd., provided the HRP-labeled anti-His tag rabbit polyclonal antibody.

2.2 Main Instruments and Reagents

Octet RED96 was supplied by Pall Fortebio Company, as was the SA sensor (lot: 1305151). The Duoflow was provided by Bio-Rad, while the enzyme immunoassay instrument was furnished by Thermo, and the biochemical analyzer was furnished by Toshiba. Latex reagents were prepared in accordance with the company's standard procedures, and the nanobody latex reagent was specifically formulated with the addition of 2.5% PEG6000 to augment the immune response.

2.3 Experimental Methods

2.3.1 SDS–PAGEgel electrophoresis identification of antibodies

The purity and size of the recombinantly expressed and purified nanobodies and rabbit polyclonal antibodies were identified using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.3.2 ELISA Detection of Antibody Affinity

Cys-C antigen at varying dilutions (100X, 200X, 400X, 800X, 1600X, 3200X, 6400X, 12800X) (original concentration: 1 mg/ml) was coated, followed by incubation with corresponding concentrations of antibodies. HRP-labeled secondary antibodies were incubated at 37°C for 40 min, followed by OPD color development, and the OD490 value was measured.

2.3.3 Biollayer interferometry (BLI) measurement of antibody affinity

Using AntiBiotin chips, nanobody Cys-VHH and polyclonal Cys-rPAB were captured. Captured antibodies were then used to bind different concentrations of antigen, followed by dissociation in buffer. Finally, the affinity kinetic constants were calculated through instrument algorithms.

2.3.4 Reagent Preparation and Determination of Calibration Curve

Nanobody Cys-VHH and rabbit polyclonal Cys-rPAB were added to our in-house latex reagent. The latex reagent was prepared according to the company's standard procedure, with 2.5% PEG6000 added to the nanobody reagent to enhance the immune response. Clinical testing was then conducted.

Calibrators of Cys-C were prepared at 0, 0.5, 1, 2, 4, and 8 mg/L by dilution. Absorbance was measured after reaction with both nanobodies and polyclonal antibodies, followed by color development, and the change in absorbance was measured. A linear curve

was plotted with calibrator concentration as the x-axis and the change in absorbance as the y-axis, and the correlation coefficient curve r was calculated.

2.3.5 Clinical Relevance Experiment

Thirty random clinical samples were tested with both reagents, and the results were statistically analyzed.

2.3.6 Precision Measurement of Reagents

High and low concentrations of two clinical samples were selected. Ten repeated measurements were conducted using both VHH nanobodies and polyclonal reagents, and the average value, standard deviation, and coefficient of variation (CV) were calculated.

3. RESULTS AND DISCUSSION

3.1 Preparation and Detection of Cys-VHH-104/704 Nanobodies

The paired nanobodies Cys-VHH-104 and Cys-VHH-704, prepared in our laboratory through expression in Escherichia coli, were verified alongside rabbit polyclonal Cys-rPAB using the SDS–PAGE method. The results exhibited distinct bands at the 20 kD mark for the nanobodies and corresponding bands of appropriate size for the polyclonals, indicating good purity of the antibodies that meet the requirements for subsequent experimental needs.

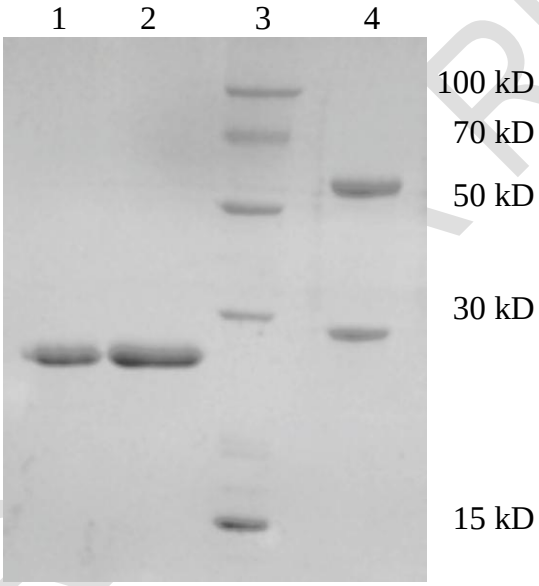


Fig. 1 Validation of recombinant antibodies
Note：1. Cys-VHH-104; 2. Cys-VHH-704; 3. Marker; 4. Cys-rPAB

3.2 Measurement of Nanobody Affinity

3.2.1 ELISA Detection of Affinity Between Different Antibodies and Antigens

The affinity between Cys-VHH-104 and Cys-VHH-704 antibodies and Cys was assessed using the ELISA method. The experimental results (Table 1) revealed the order of affinity strength as follows: Cys-rPAB > Cys-VHH-104 > Cys-VHH-704. This indicates that polyclonal antibodies indeed have their own advantages in terms of affinity, but the affinity of the nanobody Cys-VHH-104 is very close to that of Cys-rPAB.

Table 1. Protein titer determination results

Dilution factor	Group			
	Cys-VHH-104	Cys-VHH-704	Cys-rPAB	Control group
100	2.984	2.772	3.012	0.111
200	3.039	1.407**	3.245	0.079
400	2.874	0.290	3.056	0.087
800	1.553*	0.137	2.587	0.082

1600	0.362	0.123	1.684***	0.085
3200	0.285	0.153	1.244	0.074
6400	0.019	0.124	0.524	0.084
12800	0.225	0.093	0.223	0.083

** refers to the OD value corresponding to the antigen's respective dilution factor when Cys-VHH-104 reaches 1/2 OD*
*** refers to the OD value corresponding to the antigen's respective dilution factor when Cys-VHH-704 reaches 1/2 OD*
****refers to the OD value corresponding to the antigen's respective dilution factor when Cys-rPAB reaches 1/2 OD*

Table 2. Statistical results of protein potency

Antibody	Initial protein concentration	Protein concentration required to achieve 1/2 OD
Cys-VHH-104	0.73 mg/ml	0.59μg/ml
Cys-VHH-704	0.80 mg/ml	2.84μg/ml
Cys-rPAB	0.90 mg/ml	0.21μg/ml

3.2.2 Comparison of Nanobody and Rabbit Polyclonal Antibody Affinity for Antigens through BLI Experiments

By utilizing BLI to determine the relative affinities between different antibodies and their antigens, the results reveal that the affinity between antibody Cys-VHH-104 and antigen is 9.1 pM, while the average affinity between antibody Cys-VHH-704 and antigen is approximately 528.8 pM (550 pM & 507.7 pM). The affinity between the antibody Cys-rPAB and antigen is less than 1 pM. In summary, the ranking of the affinities between the three antibodies and their respective antigens is as follows: Cys-rPAB > Cys-VHH-104 > Cys-VHH-704, which aligns with the previous ELISA findings.

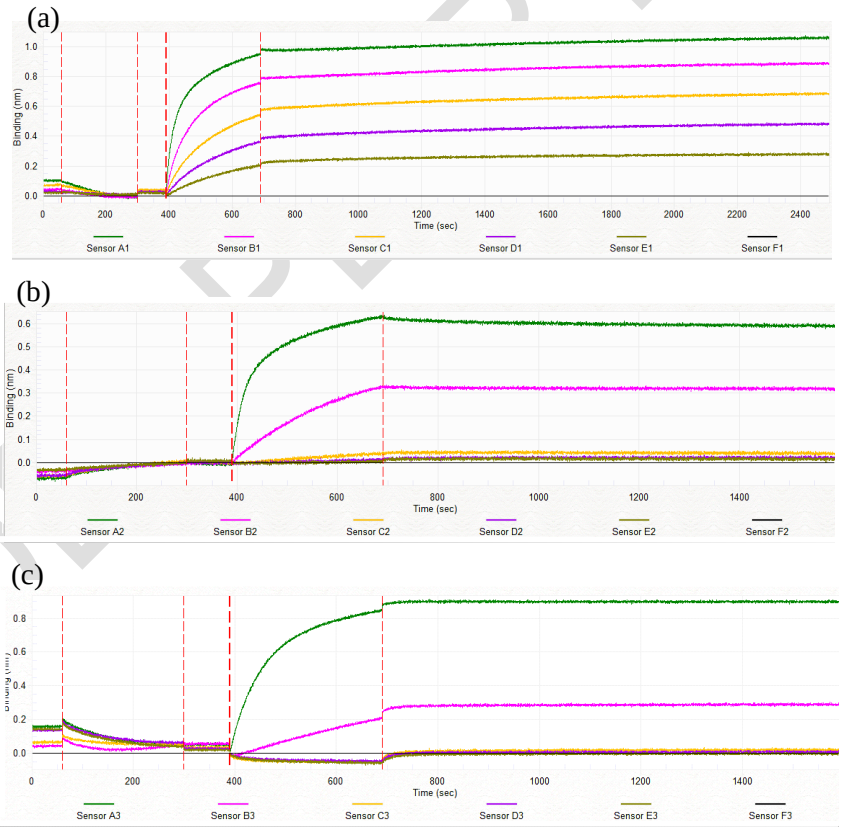


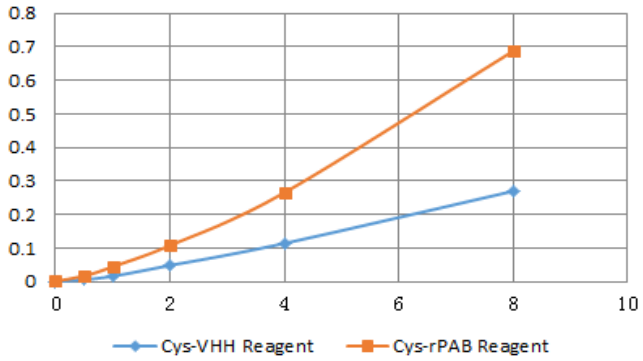
Fig. 2 Determination of antibody affinity
a) Results of the Cys-rPAB Cys antibody; b) Results of the Cys-VHH-104 antibody; c) Cys-VHH-704 antibody

3.3 Reagent Preparation and Clinical Testing

3.3.1 Reagent Preparation and Calibration Curve

The nanobody Cys-VHH and rabbit polyclonal antibody Cys-rPAB were individually incorporated into our custom-made latex reagent, followed by clinical testing. Analyzing the results, the calibration sensitivity of the rabbit polyclonal antibody Cys-rPAB appears to be superior to that of the nanobody Cys-VHH. However, both antibodies meet the requirements for normal immunoturbidimetric testing reagents.

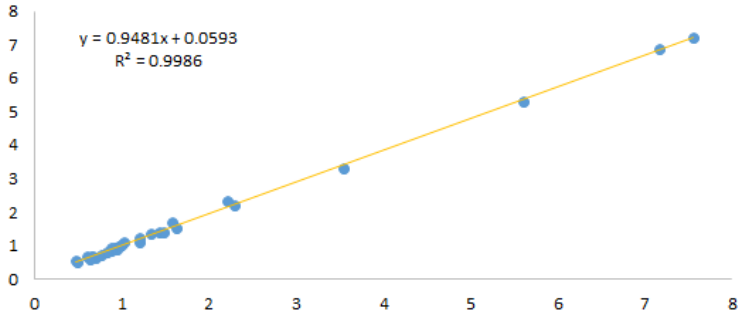
Fig. 3 Calibration Curve



3.3.2 Clinical Correlation Analysis

A statistical correlation analysis was conducted on the clinical results obtained from the reagents containing the two different antibodies. The correlation coefficient R2 was 0.9986, and the calculated results of the regression equation were found to be satisfactory.

Fig. 4 Clinical correlation analysis



2.3.3 Reagent Precision Testing

The measured values are shown in the figure below. For the Cys-VHH reagent, the coefficients of variation for the high- and low-value samples were 2.79% and 0.84%, respectively. For the Cys-rPAB reagent, the CVs for the high- and low-value samples were 2.59% and 0.69%, respectively.

Table 3 Reagent precision testing

Group		Detection Indicators		
		AV*	SD*	CV*（%）
High value samples	Cys-VHH Reagent	0.757	0.021	2.79
	Cys-rPAB Reagent	0.739	0.019	7.59
Low value samples	Cys-VHH Reagent	3.32	0.028	0.84
	Cys-rPAB Reagent	3.135	0.022	0.69

** AV: average value, SD: standard deviation, CV: coefficient of variation*

3.4 DISCUSSION

Nbs are garnering attention in disease detection due to their ease of accessibility, cost-effectiveness, and high expression levels. For example, in the diagnosis of SARS-CoV-2, Nbs are particularly suitable for binding to hidden or obscured epitopes, given their small size, making them a common choice for lateral flow tests [13]. Gai et al. isolated peripheral blood mononuclear cells from immunized

camelids for VHH library construction and screening. The selected constructs were then expressed in a suitable vector, conjugated with gold nanoparticles (AuNPs), and employed in both ELISA and lateral flow tests [14]. Initially, when recombinant antibody engineering technology emerged, with a focus mainly on scFvs, Nbs did not gain much recognition. However, accumulating research has underscored their advantages in disease detection studies [15]. In addition to the aforementioned disease detection applications, Nbs can retain functional stability under harsh chemical conditions, allowing the detection of food contaminants that induce protein denaturation through high-concentration methanol extraction [16,17].

The commercial industry has not yet extensively utilized Nb antibodies in turbidimetric immunoassay reagents, prompting this exploratory study on the application of recombinant VHH domains in turbidimetric assays. Compared to conventional antibodies, Nbs, with their smaller size and correspondingly diminished antigen recognition sites, can access areas unreachable to traditional antibodies, showing considerable potential, especially in the detection of small molecule antigens. Presently, the detection of various vitamins, residual pesticides, narcotics, and additives still heavily depends on traditional methods such as MS or HPLC, which are characterized by low throughput and slow pace [18]. By employing Nbs in turbidimetric reagents, rapid and scalable detection becomes feasible, broadening the spectrum of testing scenarios. Although the unique structure of Nbs results in affinity comparatively lower than conventional Ig structure antibodies, this study demonstrated that CYS nanobody affinity can reach 10-12 levels, an improvement over the affinities of nanobodies such as 3Nb3 (3.6 nM) and those obtained through the ADAPT platform (2 nM) [19,20]. In clinical testing of the prepared Nbs turbidimetric reagent, the precision for both low and high concentration samples was comparable to rabbit polyclonal antibody reagents, with a significant reduction in the coefficient of variation compared to conventional polyclonal antibody kits [21]. Furthermore, the correlation coefficient R2 of 0.9986 in clinical detection analysis aligns closely with previously reported values [22], substantiating the viability of Nb application in biochemical immunoturbidimetry.

4. CONCLUSION

This investigation elucidates that Nb can function as a primary component in biochemical immunoturbidimetric reagents. Although its affinity falls short of rabbit polyclonal antibodies (10-13-10-12), it achieves the pM levels typical of mouse monoclonal antibodies. In alignment with calibration curves, clinical relevance, and precision standards, it fulfills the criteria for conventional immunoturbidimetric detection reagents, indicating that Nb holds substantial prospective applicative value in biochemical reagent preparation.

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