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Authors

He, Qiyi

Yang, Li

Lin, Mingxia

et al.

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Generation of Bioluminescent Enzyme Immunoassay for Ferritin by Single-chain Variable Fragment and its NanoLuc luciferase Fusion

Qiyi He[a,b,#], **Li Yang**[c,#], **Mingxia Lin**[a], **Huiyi Yang**[a], **Xiping Cui**[a], **Mark R. McCoy**[d], **Bruce D. Hammock**[d], **Yanxiong Fang**[b,*], **Suqing Zhao**[a,*]

[a]Department of Pharmaceutical Engineering, School of Biomedical and Pharmaceutical Sciences, Guangdong University of Technology, Guangzhou 510006, People's Republic of China

[b]Faculty of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou 510006, People's Republic of China

[c]Biotherapy Center, Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, People's Republic of China

[d]Department of Entomology and Nematology and UCD Comprehensive Cancer Center, University of California, Davis, Davis, California 95616, USA

Abstract

Ferritin, widely present in liver and spleen tissue, is considered as a serological biomarker for liver diseases and cancers. The detection of Ferritin may be an important tool in health diagnosis. In this study, 14 non-immunized chicken spleens were utilized to construct a single chain fragment (scFv) phage library. After 4 rounds panning, 7 unique clones were obtained. The optimal clone was further screened and combined with NanoLuc luciferase (Nluc) as a dual functional immunoprobe to bioluminescent enzyme immunoassay (BLEIA), which was twice as sensitive as its parental scFv-based double sandwich enzyme linked immunoassay (dsELISA). The cross-reactivity analysis revealed that the proposed methods were highly selective and suitable for clinical detection. To further verify the performance of the immunoassays, serum samples were tested by the proposed methods and a commercial ELISA kit and there was a good correlation between the results. These results suggested that scFv fused with Nluc might be a powerful dual functional tool for rapid, practically reliable, and highly sensitive ferritin detection.

[*]Corresponding Authors: Prof. Yanxiong Fang, Tel.: +86 13380039529, fangyx@gdut.edu.cn; Prof. Suqing Zhao, Tel.: +86 15820258676, sqzhao@gdut.edu.cn.

[#]((Footnote))Qiyi He and Li Yang contributed equally to this work.

Declarations

All animal experimental procedures were carried out according to protocols approved by the Animal Care and Use Committee of Guangdong Province, P.R. China.

Five serum samples from healthy volunteers included 2 males and 3 females aged 25–45 were obtained from The Third Affiliated Hospital of Sun Yat-sen University which complied with The Helsinki declaration of 1975 and approved by the Guangdong University of Technology Ethics Committee.

Conflict of interest

The authors declare that there is no conflict of interests to publish this paper.

Keywords

Bioluminescent Enzyme Immunoassay; Single-chain Variable Fragment; Ferritin; NanoLuc luciferase; Biomarker

1. Introduction

Iron is an essential element for living organisms with important physiological functions and generally poor bioavailability[1]. As the primary iron storage protein, ferritin is a 450 kDa hollow nanocage capable of storing up to 4500 Fe (Ⅲ) atoms and plays a key role in iron metabolism[1]. Among them, ferritin is widely found in the liver, spleen and bone marrow[1]. The amount of iron stored in the body can be estimated by measuring serum ferritin[2]. Increased serum ferritin is an indicator of liver cirrhosis[3], liver cancer[4], ovarian cancer[5], prostate cancer[6], and atherosclerosis[7], and so on. Moreover, low amount of ferritin can lead to iron deficiency anemia[8] and malnutrition[9]. The World Health Organization (WHO) recommends a normal ferritin concentration of 30–300 ng/mL for men and 15–200 ng/mL for women. In recent decades, the detection of ferritin has been extensively studied by electrochemical immunosensor[10], radioimmunoassay[11], and inductively coupled plasma-mass spectrometry (ICP-MS)[12], etc. The instrumental methods require specific operators despite the fact they are sensitive and specific. Meanwhile, several immunoassays based on different antibody labeling and readout strategies including antibody-modified gold@carbon dot nanoconjugates immunoassay[13], Fe_3O_4 magnetic nanoparticles/chitosan composite based immunosensors[14], time-resolved fluorescence immunoassay[15], etc., have been reported.

In general, polyclonal antibodies are readily available in the serum of a variety of animals, but the quality of antibodies is uncertain and varies from batch to batch. Monoclonal antibodies solve this problem to a certain extent as they come from hybridoma cell lines with unlimited production capacity and consistency. In some cases, the peritoneal culture of monoclonal antibodies from mice is highly productive and easy to purify, but not animal friendly. Beneficial to the development of genetic tools, recombinant antibodies attracted more attention due to their small size, excellent physical performance and easy editing. Generally, the antibody library can be constructed from any species by different kinds of coding sequences. Among them, single chain fragment is one of the most popular in the recombinant antibodies. Many researchers have successfully generated scFv in different species such as mouse[16], rabbit[17], canine[18] and so on. Compared with the proposed species, the chicken derived antibodies are relatively simple by the use of only one pair of the primers of the conserved regions flanking these variable segments. Recombinant antibodies show great potential in the application of antibody-reporter fusion based on the recombinant DNA technology to achieve one-step immunoassay. Different from chemical cross-linking or nano-material modified approach, the fusion between specific antibody and signal output protein performs better in stability and consistency. Several studies have reported the application of recombinant antibody fragments with horseradish peroxidase, alkaline phosphatase as reporter proteins in the detection of drugs, biomarkers, and environmental pollutants[19–21]. The fusion antibodies show excellent performance and

is thought to be a promising probe for the establishment of biosensors. The one-step immunoassay based on fusion protein ignores secondary or tertiary-antibody conjugates, reducing operation steps and time. With smaller molecular size and higher intensity, NanoLuc luciferase (Nluc) is receiving increasing attention[22–24]. Derived from deep-sea shrimp *Oplophorus gracilirostris*, Nluc is only 19.2 kDa in size, which might be one of the smallest luciferases in the world[25]. The small Nluc is more suitable in the application of fusing with antibody considering into the steric hindrance and soluble expression of the protein. To date, there are few studies on single chain fragments of anti-ferritin, and even less on the production of fusion proteins in ferritin detection.

In this study, we prepared a single chain fragment of anti-ferritin from a chicken non-immune phage display library and then fused it to Nluc. Distinct from the immunized library, it is more important to construct a high-capacity library. To some extent, the non-immunized library reduces preparation time and is more animal friendly. As depicted in Scheme 1, ds-ELISA and BLEIA based on the scFv and its fusion protein were established respectively. Besides, the specificity and feasibility of developed methods were verified by other biomarkers and serum samples. In summary, the prepared single chain fragments against ferritin showed great potential in the detection of the human serum ferritin.

2. Material and Methods

2.1 Materials

All reagents were analytical grade unless otherwise stated. Ferritin protein (30R-3332) was purchased from Fitzgerald Industries International (Massachusetts, USA). 3,3’,5,5’-tetramethybenzidine (TMB), and isopropyl-β-D-thiogalactopyranoside (IPTG), ferritin from horse spleen were purchased from Sigma (St. Louis, MO). Alpha-fetoprotein (AFP), Carcinoembryonic antigen (CEA), C-reactive protein (CRP) were purchased from Cloud-Clone Co., Ltd (Wuhan, China). Polyethylene glycol 8000 (PEG8000) was purchased from Beijing Dingguo Changsheng biotechnology Co. LTD. (Beijing, China). Lymphocyte Separation Medium was purchased from Tianjin Haoyang Biological manufacture Co., Ltd (Tianjin, China). RNAiso Plus and PrimeScript™ 1st Strand cDNA Synthesis Kit were obtained from Takara (Dalian, China). Gel Extraction Kit, and Plasmid Mini Kit were obtained from Omega Bio-tek, Inc. (USA, Georgia). M13KO7 helper phage, SfiI and T4 DNA ligase was obtained from New England Biolabs (Beverly, MA). The phagemid vector pComb3X was a gift from Dr. Carlos F. Barbas (The Scripps Research Institute, La Jolla, CA, USA). pNLF1 vectors purchased from Promega Corporation (WI, USA). The Electrocompetent *E.coli* ER2738 cells were acquired from Lucigen Corporation (Middleton, WI). Anti-M13 Major Coat Protein Antibody (RL-ph1) HRP was purchased from Santa Cruz Biotechnology, Inc. (Texas, USA). The anti-HA tag antibody with HRP were purchased from Abcam (Cambridge, MA). B-PER and HisPur Ni-NTA resin were purchased from Thermo Fisher Scientific (IL, USA). TOP 10F’ cells were obtained from Beijing Zoman Biotechnology Co.,Ltd. (Beijing, China). Coelenterazine-h (CTZ-h) was obtained from NanoLight Technologies (Arizona, USA). Human Ferritin ELISA Kit was purchased from Shanghai Enzyme Link Biotechnology Co., Ltd. The anti-Ferritin polyclonal antibody was obtained by the immunization against the New Zealand white rabbit with

Ferritin protein in the previous work[26]. In this work, ultrapure water was used throughout the experiments.

2.2 Construction of Single-chain Variable Fragment library

All animal experimental procedures were carried out according to protocols approved by the Animal Care and Use Committee of Guangdong Province, P.R. China. 14 adult female chickens were euthanized, and their spleens were harvested. The lymphocytes were isolated from the spleens with the peripheral lymphocyte separation medium and stored at −80 °C until being used for total RNA extraction. Total RNA was prepared from lymphocytes using the RNAiso Plus following the manufacturer's instructions. First-strand complementary DNA (cDNA) was generated from total RNA using oligo(dT) primer and the PrimeScript™ 1st strand cDNA synthesis kit, according to the manufacturer's instructions. The scFv products were prepared according to the previous study[27] with the slight modification of the linker and the primers, as depicted in Table S1.

2.3 Bio-panning and Characterization of scFv Against Ferritin

The bio-panning for scFv was conducted on the basis of phage-display technology. The prepared phage-displayed scFv library was screened against ferritin. The biopanning was conducted with four rounds, as shown in Table S2. Briefly, the concentration of coating antigen was gradually reduced from 20 μg/mL, 10 μg/mL,5 μg/mL to 1 μg/mL. After blocking with 3% skim milk, 100μL/well scFv phage library was added into the microplate at 37°C for 1h. Then, the number of washes was then gradually increased round by round to remove scFvs that showed no specificity and weak binding phages. After washing, 100μL 0.1 M glycine-hydrochloric acid (Gly-HCl, pH 2.0) was added into the well and incubated for 5 min to elute binding phages, and 50 μL 1 M Tris-HCl (pH 9.0) was added to neutralize the elution buffer immediately. After four rounds of bio-panning, phage ELISA was conducted to determine the effectiveness of the bio-panning. The positive clone was extracted and sequenced for further research.

2.4 Expression and Purification of scFv

The pComb3X phagemids encoding the anti-ferritin scFvs were extracted from ER2738 and transformed into TOP 10F' cells by heat shock. A single colony was picked and cultured overnight in 10 mL of LB medium supplemented with 50 μg/mL carbenicillin. 1 mL of the overnight culture was added to 100 mL of SB containing 50 μg/mL carbenicillin. When the culture reached an OD_{600} value of 0.8, IPTG was added to a final concentration of 0.1 M, and the culture was shaken at 37 °C overnight. The culture was centrifuged, and the bacterial pellets were lysed with B-PER lysis buffer. The bacterial lysate supernatants were collected by centrifugation at 13,000 g for 10 min, followed by purification by Ni-NTA resin column. The column was equilibrated and washed with 10 mM imidazole. The captured scFvs were eluted with 250 mM imidazole. The purified scFvs were dialyzed against PBS and stored at −80 °C until use.

2.5 Construction, Expression, and Purification of scFv-Nluc Fusion Protein

The Fe38-Nluc fusion gene was constructed as following. Briefly, the Fe38 scFv gene and Nluc was amplified respectively by PCR with the primer SNF1 and SNR1, SNF2 and SNR2 (Table S1), which were added *BamHI* and *HindIII* restriction enzyme sites flanking the 3' and 5' terminal of the proposed gene. The Fe38-Nluc gene was conducted by overlap PCR with SNF1 and SNR2. The PCR products were purified and digested with *BamHI* and *HindIII*. The Fe38-Nluc gene was ligated into the fragment of digested pET22b using T4 DNA ligase, and the ligation product was transformed into the *E.coli* BL21(DE3), the positive clones were confirmed by sequencing, then the Fe38-Nluc fusion was expressed by the routine procedure.

2.6 Development of Double Sandwich Immunoassays Based on scFv or scFv-Nluc Fusion Protein

To develop the immunoassays against ferritin, a sandwich format was performed, in which anti ferritin polyclonal antibody was used as capture antibody and the Fe38 or Fe38-Nluc fusion protein as detect antibody. In a typical bioluminescence immunoassay, white 96-well microtiter plate was coated with polyclonal antibody at 100 μL /well overnight at 4 °C. The wells were blocked with 3% skim milk in PBS for 1 h at 37 °C. After washing four times with 0.01 % PBST to remove the blocking solution, a series of Ferritin (50 μL/well) was added into the well, along with 50 μL of the Fe38 or Fe38-Nluc Fusion antibody in each well. After washing five times with PBST, 100 μL of CTZ-h dissolved in the corresponding buffer was added, and the bioluminescent signal was measured immediately in one-step BLEIA by Tecan F200 microplate readers (Mannedorf, Switzerland). On the contrary, anti-HA tag antibody with HRP was added in the two-step ELISA and incubated at 37°C for another 1h. After the final washing, color development using TMB substrate (100 μL/well) was allowed to proceed for 15 min. The optical density (OD) was measured at 450 nm within 10 min after terminating the color development with sulfuric acid (50 μL/well).

2.7 Specificity

The cross-reactivity analysis was performed to detect the specificity of the proposed methods. C-reactive protein (CRP), alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), BSA and hemoglobin and ferritin from horse spleen with 1 μg/mL were prepared and tested by ds-ELISA and BLEIA for ferritin.

2.8 Validation study in the serum samples

Five serum samples from healthy volunteers included 2 males and 3 females aged 25–45 were obtained from The Third Affiliated Hospital of Sun Yat-sen University which complied with The Helsinki declaration of 1975 and approved by the Guangdong University of Technology Ethics Committee. The samples were tested by commercial ELISA kit, the proposed ELISA and BLEIA to verify the feasibility of the scFv and its fusion protein.

3. Result and Discussion

3.1 Construction of the scFv Library

Spleens were harvested from 14 chicken from the local market (Dongguan, Guangdong) and total RNA was then prepared with RNAiso Plus. cDNA was generated from RNA samples using oligo(dT) primer and the PrimeScript first-strand cDNA synthesis kit, according to the manufacturer's instructions. A set of primers was used for amplification of scFv genes. The scFv genes were obtained by amplifying VH and VL gene segments separately purified and randomly combining a secondary overlap PCR with VH and VL via a flexible linker. After the SfiI digestion, the amplified scFv genes were cloned into the pComb3X phagemid vector and then electroporated into electrocompetent ER2738 cells to construct the phage-displayed scFv library. The diversity of the library was confirmed with random colony by DNA sequencing. The initial size of the phage-displayed scFv library was estimated at approximately colony 10^8 units (cfu)/mL.

3.2 Bio-panning of phage display library

The constructed phage display scFv library was screened against Ferritin through four rounds panning. Generally, the bio-panning was conducted by coating the target antigen on a plate and then the phage displayed scFv library was incubated and rinsed. In order to obtain the high-affinity scFv, the stringency of panning was increased and shown in Table S2. The preincubated concentration of the Ferritin was gradually decreased from the 1^{st} round to 4^{th} round (20 μg, 10 μg, 5 μg, 2 μg per well). The input and output phage were calculated and depicted in Table S3. It is clear that the highest elimination rate occurs in the first round probably owing to the prepared library was derived from non-immune chicken spleens. Besides, the output of 3^{rd} round and 4^{th} round was increased revealing that the positive clones might be enriched. After 4 rounds panning, polyclonal phage ELISA was conducted to check the effectiveness and shown in Figure S1. As the number of rounds increases, positive clones are enriched and there was a specific response after the 4 rounds of panning. 40 individual clones were randomly selected from the fourth round's output, cultured and tested by phage ELISA. 23 clones were considered as positive colonies which the OD_{450} in phage ELISA was 2 times higher than blank control. 7 unique clones were selected and named as Fe01, Fe03, Fe09, Fe17, Fe24, Fe30 and Fe38 after sequencing.

Sequencing of the seven unique positive clones were confirmed by BLAST search, which are consistent with chicken VH and VL regions (Figure 1). As shown in Figure, reduced amino acid sequences contained conserved sequences in the framework regions (FRs) variable sequences in the complementarity-determining regions (CDRs) of the heavy and light chains.

3.3 scFv-based double sandwich ELISA

Among the 7 positive colonies, the plasmid Fe38 performed highest affinity by direct ELISA, was then transformed into TOP10F' cells to produce soluble protein. The crude protein was extracted by B-PER reagent and then the supernatant was collected, purified with Ni-NTA column and analyzed by SDS-PAGE. The molecular mass of the Fe38 was about 34kDa (Figure S2).

Typically, two kinds of antibody denoted as capture antibody and detection antibody were applied in double sandwich immunoassay. Due to the low background signal, polyclonal antibody with multiple epitopes and large molecular weight was selected as capture antibody in this study, and paired with Fe38 scFv as detection antibody. Prior to the ds-ELISA, the optimal concentration of the capture antibody and Fe38 scFv were determined by a checkerboard assay (Figure S3). As shown in Figure 2A, the standard curve of the ds-ELISA was established and the linear equation (Y = 0.015X+0.33, R^2=0.977) from 8 ng/mL to 80 ng/mL. The limit of detection (LOD) was 1.84 ng/mL based on 3×standard deviation of the three blank measurements/slope.

3.4 scFv-Nluc fusion protein-based BLEIA

The Nluc gene integrated with Fe38 through specific enzyme digestion and ligation. The Fe38-Nluc plasmid was transformed into pET22b for soluble fusion protein expression. Similarly, the fusion protein was obtained according the previously stated. The gel shown has a band at approximately 53kDa, thought to be the Fe38-Nluc fusion protein (Figure S2).

Coelenterazine was selected as the substrate for Nluc in this study because it is more economical and convenient than proenzyme or commercial enzymatic hydrolysis buffers. Among the CTZ analogs, CTZ-h performed better as reported by Ren et al [24]. To obtain a stable and high luminescent signal, the luminescence assay buffer was further studied. As shown in Figure S3, various buffers have different bioluminescence properties. Compared with the PBS, the introduction of surfactant additives such as tween-20 or NP-10 reduced the luminescence intensity while providing a longer half-life. BSA produces a signal and half-life similar to PBS. Besides, the 1% NP-10 buffer demonstrated longer half-life and higher signal than the commercial Glo lysis assay. Thus, we adopted PBS containing 1% NP-10 as the assay buffer for further study.

Likewise, the BLEIA was conducted under the optimization of the capture antibody and detection antibody with chessboard assay. The linear equation (Y = 233.71X+2940.69, R^2=0.981) from 2 ng/mL to 100 ng/mL was depicted in Figure 2B. The limit of detection (LOD) was 0.93 ng/mL. The proposed BLEIA not only had a lower limit of detection, but also was more advantageous in the testing time. Both scFv based immunoassays were comparably summarized in Table S4. Comparing with other methods, the proposed methods took advantage of by using the genetic antibody and its fusion antibody which was more producible and cost economic. Besides, both immunoassays achieved a lower sensitivity and had desirable potential in the detection of ferritin.

3.5 Specificity

To evaluate the specificity of the proposed immunoassays, biomarkers that can be possibly found in the human practical serum sample such as CRP, AFP, CEA, or other proteins might revolved in the panning, and heterologous ferritin were analyzed. As shown in Figure 3, only ferritin from human spleen had a signal response in the ds-ELISA and BLEIA while the others were almost negative. It is worth mentioning that although they were all ferritin, there are certain differences in protein conformation between the different sources of ferritin, resulting in unrecognizable nucleic acid sequences between the horse spleen ferritin and the

proposed antibodies. The result indicated that immunoassays based on double antibodies had high specificity to detect ferritin in actual serum samples.

3.6 Analysis of human serum samples and validation

In order to verify the feasibility of the method, ELISA, BLEIA and commercial kits were used to detect the human serum samples. The results were shown in Table 1. All three methods could detect the concentration of ferritin in serum. As shown in Figure S5, the constructed double antibody sandwich ELISA had a strong correlation with BLEIA in actual serum detection (R^2=0.993), and the correlation between ELISA and commercial kit was R^2=0.833, while the correlation between BLEIA with the commercial kit was R^2=0.700, both of which showed good correlation. The results suggested that scFv and its NLuc fusion protein based assay could detect ferritin from human serum and could be applied to disease diagnosis.

Conclusion

In this study, 7 unique clones were obtained from a chicken non-immune single chain fragments phage library. The optimal clone Fe38 was further transformed and the soluble scFv was expressed to establish a double sandwich immunoassay with the LOD of 1.84 ng/mL. Besides, the scFv was fused with Nluc as a dual functional immunoprobe to generate a one-step bioluminescent enzyme immunoassay with the LOD of 0.93 ng/mL, nearly 2 times lower than that of ds-ELISA. Highly selective immunoassays were also performed in conjunction with other biomarkers in human serum. To further validate the practicality of the immunoassays, serum samples were tested and showed good correlation with commercial ELISA kit. These results suggested that scFv-Nluc fusion might be a potential immunoprobe to specifically and rapidly determine the ferritin for disease diagnosis and prediction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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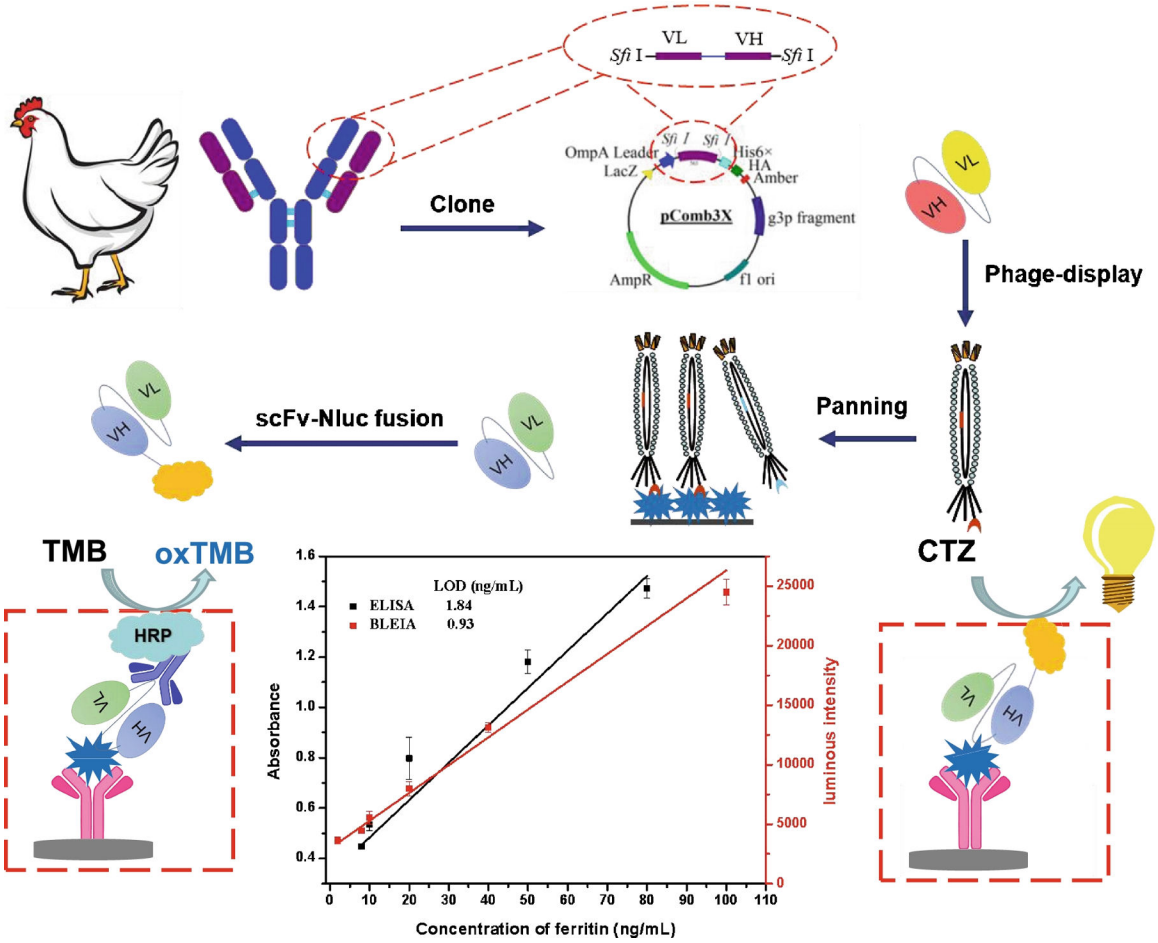

Scheme 1.

The preparation of anti-ferritin scFv and BLEIA for detection of ferritin

Figure 1.

Seven unique amino acid sequences alignment of the scFvs against Ferritin. Framework regions (FR) and complementarity determining regions (CDR) of the light chain and heavy chain are indicated below the germline sequence.

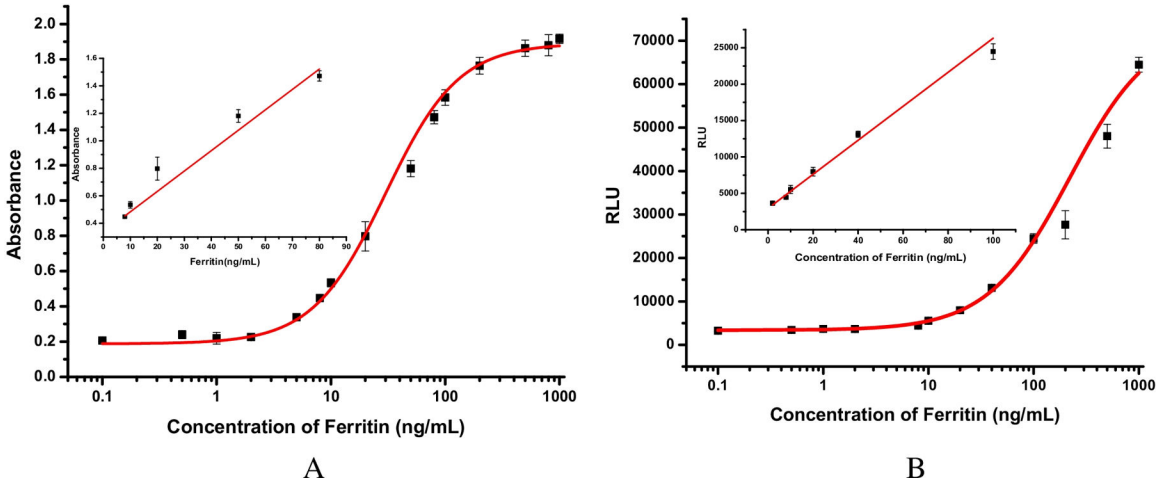

Figure 2.

Calibration plots with ELISA (A) and BLEIA (B) based on scFv Fe38 and its Nluc fusion protein, respectively. Insert were the linear relationship between the absorbance/ luminous intensity and the concentration of ferritin. Error bars indicate standard deviations (n = 3).

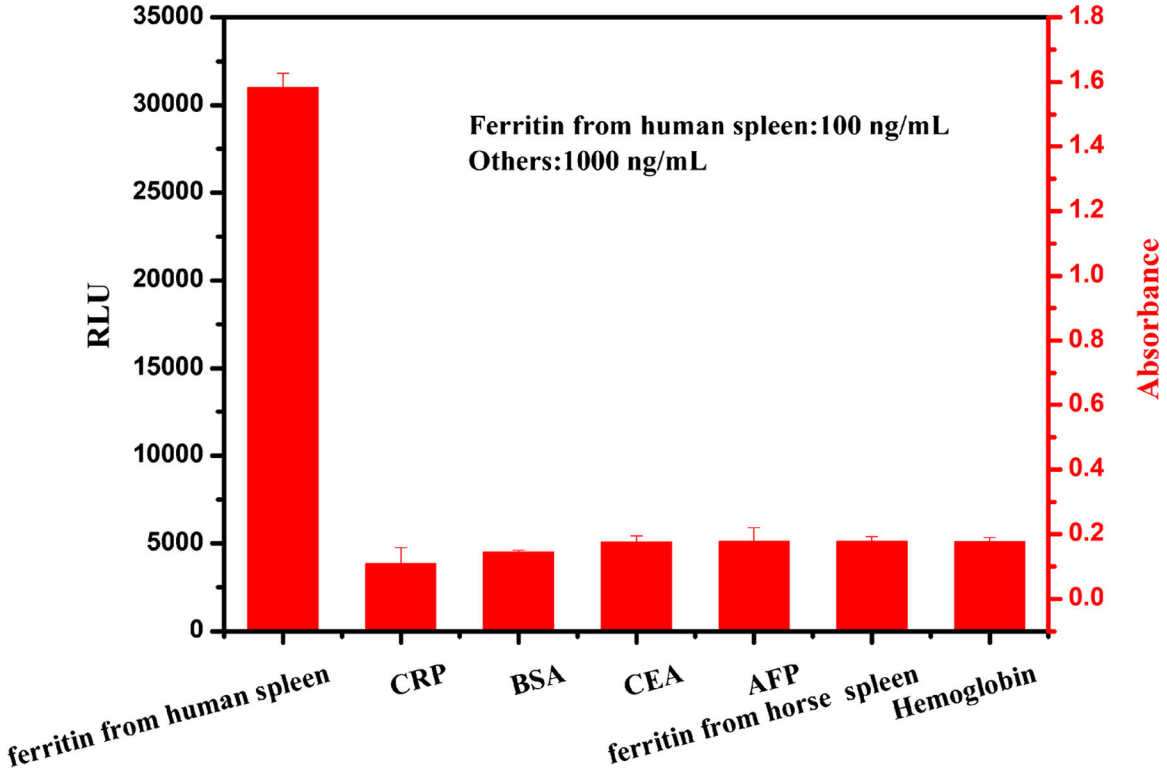

Figure 3.

Specificity of ds-ELISA and BLEIA for ferritin. The microplate was coated with 100 μL polyclonal antibody (4 μg/mL) in CBS for overnight at 4 °C. Then 100 μL analytes (100 ng/mL ferritin or 1000 ng/mL others protein) were added for 1h at 37°C. The binding was detected with Fe38 scFv (HRP labeled mouse anti-HA antibody (1/10000) as tertiary antibody) or Fe38 scFv-Nlu. Error bars indicate standard deviations (n = 3).

Table 1.

Ferritin analysis by ELISA, BLEIA and commercial ELISA kit in human serum

Samples	Concentration of Ferritin (ng/mL)		
	ELISA (*n*=3)	BLEIA (*n*=3)	Commercial kit (*n*=3)
1	109.3±7.47	107.5±2.68	119.2±5.89
2	84.79±1.86	86.84±4.52	98.75±8.83
3	123.3±1.86	125.6±6.81	144.0±15.3
4	91.65±4.11	99.61±4.77	113.7±5.30
5	120.4±2.99	115.3±2.50	132.5±11.8