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Recombinant streptavidin fusion proteins as signal reporters in rapid test of human hepatitis C virus infection

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Background: Early diagnosis of hepatitis C virus (HCV) infection is very important for the treatment of the disease. Development of sensitive and specific rapid detection assays is of great significance for the diagnosis. Here, we describe a promising method of using gold-labeled streptavidin fusion proteins as novel signal reporter in a rapid detection assay for HCV infection.

Methods: Recombinant genes encoding streptavidin fused with *Escherichia coli* maltose-binding protein (MBP) or with a portion of bacterial translational initiation factor 2 were cloned in expression vectors pMAL-5CX and pET28 and transformed in proper *Escherichia coli* host strains. The genes were induced and streptavidin fusion proteins, named M-STV and IF-STV, respectively, were purified by affinity chromatography to over 90% purity. The biotin-binding activity of M-STV and IF-STV was tested by enzyme-linked immunosorbent assay (ELISA). M-STV was labeled with colloidal gold nanoparticles and used as a signal reporter to develop a lateral flow-based rapid test for detecting anti-HCV antibodies in human blood samples.

Results: M-STV showed slightly higher biotin-binding activity and similar binding specificity as compared to commercial streptavidin. The gold-labeled M-STV bound specifically to biotin moieties immobilized on the rapid test strips in a dose-responsive manner and was successfully used in detecting HCV antibodies in serum samples of patients infected with HCV. The rapid test displayed higher detection sensitivity than gold-labeled commercial NeutrAvidin.

Conclusion: Our results indicate that gold-labeled M-STV is a promising agent in rapid tests of HCV infection and possibly other viral infections.

KEYWORDS

colloidal gold labeling, hepatitis C virus, rapid tests, streptavidin fusion proteins

1 | INTRODUCTION

Hepatitis C virus (HCV) infection is a serious health problem affecting roughly 2.2%-3% of the world's population.¹⁻³ In China, the HCV infection rate was estimated to be about 2.2%-3.7%,³⁻⁵ but like other chronic viral infections, the majority of infected patients remain undiagnosed, partly due to the asymptomatic feature of the infection until late stages of the disease progression and partly due to the lack

of resources to perform large-scale laboratory tests, especially in the rural areas in China.⁶⁻⁸ As up to now there is no effective HCV vaccine available to prevent further spread of the infection, early diagnosis and systematic screen of high-risk population will have several advantages over late-stage diagnosis. Early diagnosis not only gives infected people time and opportunity to adjust their lifestyle to delay and slow down the disease progression, but also gives the doctors more therapeutic options to choose to treat the disease.⁹ In

addition, early diagnosis will also let people be aware of the disease and take necessary measurement to prevent further infections.¹⁰

The routine diagnosis of HCV infection involves detecting anti-HCV antibodies in the blood, typically by enzyme-linked immunosorbent assay (ELISA), which requires laboratory equipment and trained medical technicians. Recently, lateral flow chromatographic rapid test based on HCV antigen-antibody interactions has been developed and used in point-of-care clinical testing.^{11–14} In these rapid tests, protein A or HCV antigens labeled with colloidal gold nanoparticles were used as detection reporters. These reporter molecules have high binding affinity and specificity toward the anti-HCV antibodies captured by the HCV antigens on the solid phase, thus ensuring high sensitivity and high specificity of the rapid test devices. The rapid tests open a new opportunity to increase the diagnosis rate of HCV in places where more sophisticated laboratory testing is not available and where large-scale HCV screens are required.

Besides protein A and specific antigens, another molecular interaction widely used in biological detection is between streptavidin and biotin.^{15,16} The binding between streptavidin and biotin is noncovalent but has extremely high affinity, with the dissociation constant of about 10^{-15} mol/L, about 10^3 – 10^6 times higher than that of typical antigen-antibody interaction.¹⁵ The biotin-streptavidin and biotin-NeutrAvidin, a deglycosylated form of avidin,¹⁷ have also been used in rapid tests.^{18–20} In this report, we describe construction of two streptavidin fusion proteins, labeling them with colloidal gold and using them in a rapid test system for HCV detection. The rapid test using the gold-labeled streptavidin fusion protein was more sensitive than using the commercial gold-labeled streptavidin products as the detection reporter.

2 | MATERIALS AND METHODS

2.1 | Constructing recombinant genes encoding streptavidin fusion proteins

The 477 bp streptavidin coding sequence (*stv*) without the signal peptide region (Genbank accession #: X03591) was synthesized at Sangon Biotech (Shanghai, China).²¹ The *stv* was amplified by PCR using primer pair STV-HIS (forward, 5'-ACACCATATGGGCATCACC GGCACCTGGTA; reverse, 5'-ACAGGATCCTTAGTGGTGATGATGGT GATGCTGCTGAACGGCGTCGAGC) to introduce 6 histidine codons (HIS-tag) at the 3'-end of the gene. The *stv* PCR product was digested with restriction enzymes NdeI and BamHI, and cloned into expression vectors pMAL-5cx (New England Biolabs), which allowed in-frame fusion between *stv* and the gene encoding maltose-binding protein (MBP) on the vector. The pMAL-5cx::stv recombinant plasmid was transformed into *E. coli* ER2523 and selected on LB agar plates containing ampicillin (100 µg/mL).

The *stv* PCR product was also cloned into pET28a (Novagen, Darmstadt, Germany), and the pET28a::stv recombinant plasmid was transformed into *E. coli* strain DH5α by selecting on LB agar plates containing kanamycin (50 µg/mL). To construct IF2-streptavidin fusion

protein (IF-STV), a portion of *E. coli* translational initiation factor II gene encoding amino acids #159–390 was amplified by PCR using the primer pair IF-2 (forward, 5'-ACACTCATGAGCAATCAACAAGACGATA; reverse, 5'-ACACCATATGGCGCGGTTTCAGCCGCAGC). The PCR product was digested with BspHI and NdeI and cloned into the pET28::stv digested with NcoI and NdeI. The recombinant plasmid was transformed into strain BL-21 by selection on kanamycin (50 µg/mL) and was referred to as pET28::IF-STV.

2.2 | Purification of recombinant proteins

To express recombinant streptavidin fusion proteins, cells were grown in LB at 37°C and, at OD₆₀₀ = 0.3, expression of the fusion proteins was induced by adding IPTG to a final concentration of 0.1 mmol/L. The cells were let grown for 3 more hours and then harvested by centrifugation. The cells were resuspended in 50 mmol/L Tris-HCl buffer (pH 7.5) containing 100 mmol/L NaCl, lysed by sonication, and centrifuged at 15 000 g for 30 minutes at 4°C.

To purify M-STV from cells carrying pMAL-5cx::stv, the supernatant was passed through an amylose resin column (New England Biolabs, Hitchin, UK), rinsed, and eluted according to the manufacturer's instructions. The fractions containing recombinant proteins were pooled and loaded to a HisPur Cobalt Spin Column (Thermo Scientific, Rockford, IL, USA) for purification targeting the HIS-tag at the C-end of the fusion protein. The column was equilibrated, loaded, rinsed, and eluted according to the manufacturer's instructions. Fractions containing purified recombinant proteins were pooled and dialyzed (molecular weight cutoff = 10 k) twice against 1 L of 20 mmol/L phosphate buffer, pH 7.0. For purification of recombinant HIS-tagged IF-STV from cells carrying pET28a::IF-STV, the supernatant was directly loaded onto the HisPur Cobalt Spin Column, and the HIS-tagged IF-STV was purified as described above. To monitor the purification steps and estimate the purity of the purified proteins, SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 10%–20% gradient gels.

2.3 | ELISA tests

2.3.1 | Binding efficiency test

Recombinant streptavidin fusion proteins as well as streptavidin from a commercial source (Thermo Scientific) were diluted to 17 nmol/L in 0.1 mmol/L NaHCO₃, pH 8.4, and 100 µL was used to coat 96-well EIA/RIA plates (Corning, Corning, NY, USA). The plates were placed at 4°C overnight. All the subsequent steps were carried out at room temperature unless specifically indicated. The unbound proteins were removed, and the wells were washed 3 times with 360 µL of phosphate-buffered saline (PBS, 10 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.4) containing 0.05% Tween-20 (PBST). The wells were blocked with 360 µL of 5% nonfat milk (NFM) in PBS for 1 hour. The blocking solution was removed from the wells, and 100 µL of biotin mouse anti-human IL-1α monoclonal antibody (BioLegend, San Diego, CA, USA) serially diluted in 2% NFM in PBS was added. The binding was allowed to proceed for 1.5 hours.

The wells were emptied and washed three times with PBST. Then 100 μ L of HRP goat anti-mouse IgG (BioLegend), diluted 1:1000 in 2% NFM in PBS, was added and incubated for 0.5 hours. The wells were emptied and washed three times with PBST, and 100 μ L of TMB Substrate (Thermo Scientific) was added. The plate was incubated at 37°C for 10 minutes. The color development reaction was stopped by adding 50 μ L of 2N HCl and A_{450} was measured. Dose-responsive curves were generated after subtracting the blanks.

2.3.2 | Binding specificity test

Wells of 96-well EIA/RIA plates were coated with 380 μ L of 5% NFM in 0.1 mol/L NaHCO_3 , pH 8.4 at 4°C overnight. The wells were emptied and rinsed 3 times with 360 μ L of PBST. Then, 100 μ L of recombinant streptavidin fusion proteins and commercially obtained streptavidin, NeutrAvidin®, and Avidin (all from Thermo Scientific), diluted to 17 nmol/L in PBS, were added to the wells and incubated for 1.5 hours. The wells were emptied and washed three times with PBST. The biotin mouse anti-human IL-1 α monoclonal antibody (1 μ g/mL) was added. The binding was allowed to proceed for 1.5 hours. The subsequent steps of washing, adding HRP Goat Anti-mouse IgG, and adding TMB Substrate for color development were the same as described in the binding efficiency test above. For each protein tested, four wells were coated and the results were averaged after subtracting the blanks.

2.4 | Preparation of colloidal gold particles and labeling of proteins

To 100 mL of triple distilled water in a beaker, 1 mL of 1% chloroauric acid (HAuCl_4) was added and mixed. The beaker was heated with moderate stirring until boiling, and 1.5 mL of 1% trisodium citrate was quickly added with increased stirring while the heating was continued. When the gold nanoparticle suspension formed and gradually turned to wine-red, the heating was stopped. The stirring was continued till the solution was cooled to near room temperature. Water was added to compensate the lost water volume during the heating process.

To prepare gold-conjugated M-STV and NeutrAvidin, 240 μ L of 1% potassium carbonate was added to 30 mL of the colloidal gold suspension and mixed well. Based on the results of a preliminary optimization test, 120–160 μ L of proteins (1 mg/mL in water) was added slowly to the gold suspension with stirring. After mixing, the suspension was let sit at room temperature for 10 minutes. Then, 10% bovine serum albumin (BSA, Sigma-Aldrich, Darmstadt, Germany) was added to a final concentration of 0.1% and let sit for 5 minutes at room temperature. The suspension was centrifuged for 30 minutes at 10 000 g. The supernatant was carefully removed by aspiration, and 1.5 mL of 2 mmol/L borax buffer containing 0.1% BSA and 10% sucrose was added. The resuspended colloidal gold was stored at 4°C.

2.5 | Preparation of lateral flow rapid test strips

The lateral flow strips for rapid test were designed and assembled following the general outlines described as Chiao et al.²² A

recombinant DNA fragment, encoding the first 50 amino acids of the core protein and 267 amino acids of the NS3 region of HCV genome, was cloned in pET28a, and the 3'-end was fused in-frame with the HIS-tag coding sequence on the vector. The recombinant plasmid was transformed into *E. coli* BL-21. The recombinant HCV protein was induced and HIS-tag purified as described above. This HCV antigen protein was used in two ways: (a) It was labeled with biotin using Sulfo-NHS-LC-Biotin (Thermo Scientific) following the manufacturer's instruction; and (b) In its unlabeled form, the HCV antigen was dispensed on the nitrocellulose (NC) membrane to generate the "test line," serving as the capture agent in the test. The biotin mouse anti-human IL-1 α monoclonal antibody was also dispensed on the NC membrane to generate the "control line." The protein dispensing on the NC membrane was performed using Precision Sprayer HGS510 (Autokun, China). After the dispensing of test line and control line, the NC membrane, together with the sample pad and the absorbance pad, was attached to the solid support and cut into test strips of 3 mm wide using Autokun's Guillotine Cutter HGS210.

2.6 | Rapid test of serum samples using the test strips

The rapid test was carried out in 2-mL Eppendorf tubes. In general, the following reagents were added to each 2-mL tube: 20 μ L of human serum (HCV-positive or HCV-negative), 10 μ L of assay mixture (containing sucrose, BSA, sodium citrate, CHAPS, Triton X-100, and mouse IgG), 10 μ L of gold-conjugated M-STV or gold-conjugated NeutrAvidin, and, for detecting HCV, 2 μ L of properly diluted biotin-conjugated HCV antigen. Immediately after the reaction mixture ingredients were combined and mixed, the rapid test strips were placed vertically into the 2-mL tubes to start the lateral flow chromatography. The results were recorded 10 minutes after the start.

In the dose-response test, varying amounts of gold-labeled M-STV (1, 2, 4, 6, 8, or 10 μ L) were added to the 2-mL tubes. In the assay specificity test, 5 μ g of unlabeled M-STV, BSA, and MBP or 1 μ g of biotin mouse anti-human IL-1 α monoclonal antibody was added to the tubes.

To compare the detection sensitivity between the gold-labeled M-STV and gold-labeled NeutrAvidin, a HCV-positive serum sample (kindly provided by Biosino Lab Tech, Beijing, China) was diluted 10-, 50-, 100-, 200-, and 400-fold with a HCV-negative human serum and added to the reaction mixture for detection.

3 | RESULTS AND DISCUSSION

3.1 | Cloning streptavidin fusion genes and purification of proteins

The coding sequence of streptavidin gene without the signal peptide portion was fused to the *malE* gene encoding MBP on the pMAL-5cx vector. HIS-tag was added to the 3'-end of the gene so the recombinant protein could be purified on affinity columns targeting MBP

and HIS-tag, respectively. The fusion protein M-STV was first purified through an amylose column, generating the M-STV of about 90%-95% purity, which was subject to a second-step purification on a cobalt resin column to generate about 95%-98% purity (Figure 1A).

It was reported that streptavidin fused to a portion of bacterial translational initiation factor IF2 gene increased solubility of streptavidin.²³ To test the effect of fusion of streptavidin with this gene, a portion of the gene encoding amino acids #159-390 was fused in-frame to the HIS-tagged *stv* gene on pET28 to produce the fusion protein IF-STV. Purification of IF-STV using affinity chromatography targeting the HIS-tag resulted in about 90%-95% purity (Figure 1B).

3.2 | Characterization of recombinant fusion streptavidin by ELISA tests

To test how efficiently the recombinant M-STV and IF-STV fusion proteins bind to biotin, an ELISA was performed using the fusion proteins to coat 96-well plates and measuring the binding of a biotin-labeled mouse monoclonal antibody to the coated fusion proteins.

FIGURE 2 Test of binding efficiency of M-STV and IF-STV to biotin-labeled antibodies. M-STV, IF-STV, and streptavidin were used to coat the EIA wells, serially diluted biotinylated mouse anti-human IL-1 α monoclonal antibody was allowed to bind to the coated proteins, and then HRP goat anti-mouse IgG was added. The binding was measured by measuring A450 after addition of HRP substrate TMB

Figure 2 shows that both M-STV and IF-STV generated higher binding signals than the commercial streptavidin used as control across the range of concentrations tested. When the biotin-labeled antibody was diluted to 0.19 ng/mL, little A₄₅₀ signal was generated by the commercial streptavidin, while M-STV and IF-STV still showed significant A₄₅₀ readings. When the biotin-labeled antibody was decreased to 0.048 ng/mL, M-STV still produced A₄₅₀ reading close to 0.2 under the test conditions, while IF-STV and streptavidin were near the detection limit. These results indicate that the recombinant fusion streptavidin proteins, especially M-STV, were better than the commercial streptavidin products in sensitivity of binding to biotinylated proteins.

The specificity of binding is an important issue in using the avidin-biotin system for molecular detection. Avidin, usually purified from chicken egg white and the first of its kind discovered, is a basically charged glycoprotein and exhibits nonspecific binding due to the glycosylation, which greatly limits its use in molecular biological detection systems.^{24,25} The binding specificity of M-STV and IF-STV was measured and compared to that of commercially acquired streptavidin, avidin, and NeutrAvidin. These proteins were tested for nonspecific binding to microtiter wells coated with excessive nonfat milk. Figure 3 shows that M-STV, IF-STV, streptavidin, and NeutrAvidin all displayed similar levels of nonspecific binding to NFM, significantly lower than avidin. Considering the higher binding sensitivity of M-STV to biotin-conjugated proteins over IF-STV and M-STV being more stable than IF-STV in solutions, M-STV was selected for further tests.

3.3 | Using recombinant fusion streptavidin in rapid test format

Our goal was to test whether the fusion streptavidin can be used in rapid test to detect specific antibodies or antigens in a biological sample such as testing hepatitis C virus (HCV) infection. For this purpose, we prepared gold nanoparticle suspensions, labeled M-STV, and used it as a signal reporter to detect biotin-conjugated antigen captured on a lateral flow-based rapid test strip. As expected, the

FIGURE 3 Test of binding specificity of M-STV and IF-STV to NFM. M-STV, IF-STV, streptavidin, NeutrAvidin, and avidin were allowed to bind to wells coated with 5% NFM. Biotinylated mouse anti-human IL-1 α monoclonal antibody was added, and then HRP goat anti-mouse IgG was added. The binding was measured by measuring A450 after addition of HRP substrate TMB

gold-labeled M-STV bound to biotin-conjugated protein printed on the rapid test strips in a dose-responsive manner (Figure 4A). The binding was not affected by the addition of BSA and MBP in the reaction mixture but was greatly diminished when unlabeled M-STV, which could compete with the gold-labeled M-STV in binding to the immobilized biotin, was added (Figure 4B). Furthermore, adding biotin-conjugated protein into the reaction mix decreased the binding of gold-labeled M-STV to an undetectable level (Figure 4B), indicating that the binding was specifically toward biotin moieties on the immobilized proteins.

To detect anti-HCV antibodies in HCV-infected patient blood, a recombinant HCV protein was constructed and purified. The protein was printed onto the nitrocellulose membrane, which was used to capture anti-HCV antibodies in the serum samples of HCV-infected patients. A part of the HCV protein was conjugated to biotin and was included in the reaction mixture that also contained the gold-labeled M-STV,

FIGURE 4 Testing the binding of gold-labeled M-STV to immobilized biotin-labeled protein. A, Dose-response of gold-labeled M-STV. Different amounts of gold-labeled M-STV from 1 to 10 μ L as indicated were run through the rapid test strips. Arrow C, the control line printed with biotin-labeled mouse anti-human IL-1 α antibody; arrow T, the test line printed with unlabeled recombinant HCV antigen. B, Competition of gold-labeled M-STV by addition of 5 μ g of different proteins: Strip 1, no protein added; strip 2, M-STV; strip 3, BSA; strip 4, MBP; strip 5, biotin-labeled mouse anti-human IL-1 α antibody. To all the strips, 2 μ L of gold-labeled M-STV was added. Arrows C and T are the same as described in A

FIGURE 5 Detecting anti-HCV antibodies in a HCV-infected serum sample using gold-labeled M-STV and NeutrAvidin on lateral flow rapid test strips. A, Detection using gold-labeled M-STV. B, Detection using gold-labeled NeutrAvidin. Strips 1, 2, 3, 4, and 5: HCV-infected serum diluted 10-, 50-, 100-, 200-, and 400-fold with negative human serum, respectively; strip 6, negative human serum only. Arrow C, the control line printed with biotin-labeled mouse anti-human IL-1 α antibody; arrow T, the test line printed with the unlabeled recombinant HCV antigen

the serum to be tested, and other supportive materials. If the serum sample is from a HCV-infected patient, the anti-HCV antibodies in the serum would be captured by the HCV antigen on the nitrocellulose membrane (the test line). As bivalent molecules in binding their specific antigens, the captured antibody would bind to the biotin-conjugated HCV protein and immobilize it. The biotin moiety would serve as a capturing agent to bind to gold-labeled M-STV and display the signal.

Our preliminary results showed that gold-labeled M-STV, similar to gold-labeled NeutrAvidin, was able to specifically detect a HCV-infected serum sample in rapid test. Then, 100 additional HCV-positive serum samples collected from several local hospitals were tested and all showed clearly positive signal on the rapid test strips using the gold-labeled M-STV. These 100 serum samples were also tested using gold-labeled NeutrAvidin, and 95 samples generated clearly positive signals, while the remaining five produced faint positive signals too weak to make a decisive judgment. Meanwhile, 100 serum samples from uninfected individuals were tested on rapid test strips using the gold-labeled M-STV and gold-labeled NeutrAvidin, respectively. Concordant results were obtained between the two detection agents, with 98 serum samples were clearly negative while the remaining 2 showing positive signals. The two positive serum samples were subsequently tested negative by PCR (data not shown) using HCV-specific primers. Taken together, the above results showed that M-STV was able to specifically detect HCV-infected serum samples, showing very low, if any, false-negative rate and low (~2%) false-positive rate.

To compare the sensitivity of detection between M-STV and NeutrAvidin, a HCV-positive serum sample was serially diluted with HCV-negative human serum. The gold-labeled NeutrAvidin, generates detectable signal when the HCV serum was diluted up to 100-fold. On the other hand, the gold-labeled M-STV showed an even better detection sensitivity as it was able to generate a clearly visible signal even at 200-fold of serum dilution (Figure 5A,B).

Fusion proteins have wide applications in modern biological research. MBP is one of the best studied and most frequently used fusion tags. The MBP moiety facilitates the purification of the target protein. In many cases, it increases the yield of their passenger proteins and helps to enhance the solubility and stability of the fusion partners.²⁶⁻²⁸ In this study, the M-STV was easily purified to over 90% purity by a single-step affinity chromatography and showed great solubility and stability. Moreover, M-STV offers more binding sites for gold labeling while not affecting the binding between the streptavidin moiety and biotin. Possibly, these features of M-STV make it a better detection reporter with higher sensitivity than streptavidin and NeutrAvidin in the rapid test.

Taken together, our results showed that gold-labeled M-STV is a promising detection agent in lateral flow-based rapid tests. Furthermore, M-STV, as novel signal reporter, has a variety of potential applications in the field of molecular detection.

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