9

Assays for Selection of Single-Chain Fragment Variable Recombinant Antibodies to Metal Nanoclusters

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Summary

The protocols herein describe colony-lift and fluorescent immunoassays that were used to identify bacterial colonies that produced single-chain fragment variable (ScFv) recombinant antibodies reactive with zero-state silver. A large (approx 2.9-billion member) phage-displayed antibody library was panned against zero valent silver. Bacterial colonies obtained after two rounds of selection were either lifted onto nitrocellulose filters or picked to individual wells of 384-well microtiter culture plates. Colonies lifted onto filters were placed onto zero valent silver-coated filters and induced to produce soluble ScFv antibodies. ScFv antibodies, expressed by individual colonies, that bound to silver nanocluster-coated filters were detected using an anti-ScFv antibody conjugated to horseradish peroxidase and a chemiluminescent substrate. Colonies picked to 384-well micotiter culture plates were induced to express soluble ScFv antibodies. ScFv antibodies in bacterial periplasmic extract were transferred from 384-well culture plates to 384-well assay plates containing zero-state silver particles and an anti-ScFv antibody conjugated to a fluorescent dye. ScFv antibodies, expressed by individual bacterial clones, that bound to zero valent silver nanoparticles in 384-well assay plates were detected using an FMAT 8100 fluorescent plate reader. The colony-lift and fluorescent immunoassays detected ScFv antibodies reactive with zero valent silver. Similar assay formats should also be useful to detect bacterially expressed recombinant antibodies or proteins to other nanoclusters.

Key Words

Single-chain fragment variable (ScFv); recombinant antibodies; horseradish peroxidase; Anti-E tag antibody; silver-coated membrane; nanoparticles.

1. Introduction

The design of readily programmable, structurally well-defined biological interfaces for inorganic materials represents a significant challenge toward realizing the promise of bionanotechnology. Encouraging approaches have used

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oligonucleotide-functionalized nanoparticles ***(1)***, genetically engineered viruses ***(2–4)***, the iron-storage protein ferritin ***(5,6)***, and several classes of polypeptides inspired by naturally occurring biomineralization systems ***(7,8)***. Additionally, several reports have demonstrated the identification of effective nanoparticle-forming peptides via combinatorial means including phage display ***(9)***, rolling mutagenesis ***(10)***, and synthetic spatially addressable libraries ***(8)***.

Recombinant antibodies reactive with antigens, silver nanoparticles, and so on can be detected using a variety of different assays. The following protocols describe two such assays that were used to detect single-chain fragment variable (ScFv) recombinant antibodies reactive with silver nanoparticles. The "silver nanoparticles" used in these assays were actually aggregates of nanoparticles. Because the purpose of these assays was to detect ScFv antibodies reactive with zero-state silver metal free of any organic or inorganic surface-bound molecules, the particles were not treated with reagents to obtain true nanoparticles.

The ScFv recombinant antibodies used in the assays were cloned into the Amersham-Pharmacia pCANTAB5E phagemid expression vector to produce a recombinant phage antibody library that contained approx 2.9×10^9 members. The ScFv antibodies assayed were obtained from bacterial isolates that stemmed from two to three rounds of phage antibody library selection on silver nanoparticles. ScFv antibodies expressed by *Escherichia coli* using pCANTAB5E display a short peptide tag (E-tag) recognized by the Anti-E tag monoclonal antibody (MAb). The Anti-E tag MAb can be conjugated to or labeled with various reporter molecules such as horseradish peroxidase (HRP) or fluorescent dyes such as FMAT™ Blue Monofunctional Dye. The HRP- or dye-conjugated Anti-E tag antibody will bind to any E-tagged ScFv antibodies that interact with silver nanoparticles in an assay. The HRP conjugated to the Anti-E tag antibody can be used to catalyze a substrate reaction to produce a color- or light-emitting (chemiluminescent) signal that can be detected using a microtiter plate reader, film, or light-capturing device. The fluorescent dye conjugated to the Anti-E tag antibody can be detected with a fluorescence microtiter plate reader. If the HRP or dye reporter molecules are present when the assay is read, then the Anti-E MAb is bound to E-tagged ScFv antibodies that interact with silver nanoparticles.

In the colony-lift assay described in **Subheading 3.1.** (**Fig. 1**), bacterial colonies that contain and express E-tagged ScFv antibodies are placed onto nitrocellulose membranes and set on bacterial culture medium ***(11)***. The colony membranes are subsequently placed on top of a silver nanoparticle-coated membrane permeated with bacterial culture medium that induces bacterial ScFv expression. ScFv antibodies expressed by the bacterial colonies diffuse through the colony filters and onto the silver nanoparticle-coated membrane. After incubation, the silver nanoparticle-coated membrane is washed to remove unbound ScFv

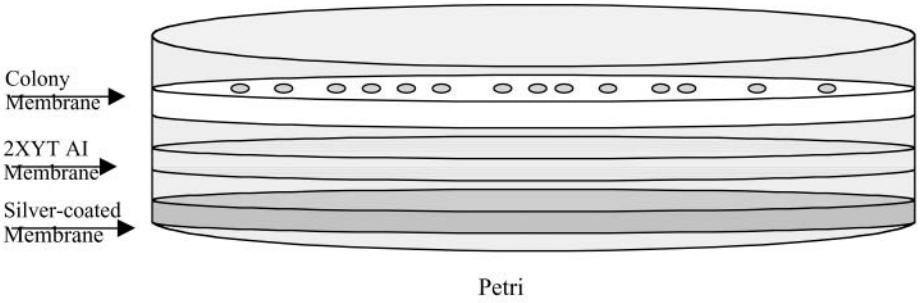

Fig. 1. Schematic diagram of a colony lift on silver-coated nitrocellulose. The silver-coated membrane is placed top side up on the bottom of a Petri dish followed by the 2XYT AI-moistened membrane and the colony-containing membrane.

antibodies and probed with Anti-E tag antibody conjugated with HRP (Anti-E/HRP). Unbound Anti-E/HRP is removed by washing. The membrane is then exposed to an HRP chemiluminescence substrate that emits a light signal that produces a black spot on photographic film wherever the Anti-E/HRP binds to ScFv antibodies that interact with silver nanoparticles. The colony-membrane and the photographic film can then be aligned to determine which bacterial colonies express ScFv antibodies reactive with silver nanoparticles.

The Applied Biosystems FMAT™ 8100 HTS System is a microtiter plate reader that uses a microscopic lens to focus on and detect a fluorescent signal present only on the bottom of a well in a microtiter plate. In the assay described in **Subheading 3.2.**, the silver nanoparticles are combined in a microtiter well with Anti-E antibody conjugated to FMAT Blue and ScFv antibodies in bacterial culture medium. The silver nanoparticles settle to the bottom of the well on incubation. The FMAT 8100 is then used to detect FMAT Blue Anti-E bound to ScFv antibodies that have interacted with silver nanoparticles present on the bottom of the microtiter wells.

2. Materials

1. 2XYT bacterial broth culture medium: Dissolve 5 g of NaCl, 10 g of Bacto™ Yeast Extract, and 17 g of Bacto™ Tryptone in 1 L of double-deionized water. Filter the medium into sterile disposable plastic bottles using a peristaltic pump and a 0.2-µm filter device. The medium can be stored at room temperature for 6–12 mo.
2. 2XYT AG agar medium: Add 15 g of granulated agar to 1 L of bacterial culture medium in a 2-L Erlenmeyer flask. Autoclave at 121°C, 15 psi for 15 min to dissolve the agar. Allow the agar medium to cool to 45°C. Add 20 g of D(+)-glucose and 100 mg of ampicillan and mix. Pour approx 30 mL of agar medium into each of 30 sterile 150 × 15 mm plastic Petri dish. Allow the agar medium to cool

overnight, and store at 4°C until needed in a closed plastic bag or container. Plates can be stored at 4°C for 1 mo.

3. 2XYT AI medium: Add 100 mg of filter-sterilized ampicillin and 238.31 mg of filter-sterilized isopropyl-β-D-thiogalactoside to 1 L of sterile bacterial culture medium. Store at 4°C until needed. The medium can be stored at 4°C for 1 mo.
4. 2XYT AG with glycerol: Add 16 mL of a 50% (v/v) filtered glycerol solution to 34 mL of 2XYT AG, mix, and then store at 4°C. This medium can be stored at 4°C for 1 mo.
5. 1X TES: To a 1-L graduated cylinder add 171.15 g of sucrose; 200 mL of 1 *M* Tris, pH 8.0; and 1 mL of 0.5 *M* EDTA. Add double-distilled deionized water to 1 L, and then mix. To prepare 1/5X TES, add 200 mL of 1X TES and 800 mL of double-deinonized water to a 1-L graduated cylinder and mix. Filter-sterilize 1X TES and 1/5X TES using a 0.2-µm filter unit. Store at 4°C.
6. Phosphate-buffered saline (PBS): To a 1-L graduated cylinder, add 1.44 g of sodium phosphate dibasic anhydrous, 8 g of sodium chloride, 0.2 g of potassium chloride, and 0.24 g of potassium phosphate. Add double-distilled deionized water to 1 L, and then mix to dissolve.
7. 0.1% Tween in PBS (PBS/T): Add 1 mL of polyoxyethylene-sorbitan monolaurate (Tween-20, Sigma cat. no. P-7949, St. Louis, MO) to 1 L of PBS and mix.
8. 0.1 *N* Nitric acid in double-deionized water.
9. Silver, nanosize-activated powder (Aldrich cat. no. 48,405-9).
10. HRP/Anti-E tag conjugate (Amersham cat. no. 27-9413-01).
11. FMAT Blue Monofunctional Dye/Anti-E tag conjugate. Prepare conjugate according to the manufacturer's instructions using the Anti-E Tag Antibody (Amersham cat. no. 27-9412-01) and an FMAT™ Blue Monofunctional Dye kit (Applied Biosystems cat. no. 432851).
12. 384-Well black, clear-bottomed microtiter plates for use with the FMAT 8100 HTS System (Applied Biosystems cat. no. 4315481).
13. 384-Well polystyrene plates.
14. 384-Pin polypropylene replicator.
15. Protran® pure nitrocellulose transfer and immobilization membranes (132-mm, 0.45-µm pore size) (Schleicher and Schuell cat. no. 10402525).
16. SuperSignal® West Pico Chemiluminescent Substrate (Pierce cat. no. 24080).
17. Scientific imaging film and cassette (8- × 10-in).
18. Required instrumentation: multichannel pipet, 30°C incubator, film developer, FMAT 8100 HTS System, and clinical centrifuge equipped with microtiter plate carriers.

3. Methods

3.1. Colony Lift

1. Day 1: Spread bacteria to obtain individual colonies to be assayed for ScFv activity on 2XYT AG plates (*see* **Note 1**). Invert the plates and incubate at 30°C overnight in a humidified container.

2. Day 2: Use a multichannel pipet to add 75 µL of 2XYT AG medium to each well of a Nunc 384-well plate.
3. Aseptically pick or transfer individual colonies from the 2XYT AG agar plates to individual wells of the 384-well plate containing 2XYT AG medium. Incubate the plate at 30°C overnight in a humidified container. This is the master plate.
4. Day 3: Use a pen or pencil to mark a 132-mm nitrocellulose membrane so that it can be oriented later on. The side of the membrane marked will be the top side of the membrane.
5. Use a 384-pin replicator to transfer a small amount of bacterial culture medium from each well of the 384-well master plate to the top side of the 132-mm nitrocellulose membrane. Wrap the 384-well master plate in plastic wrap and store at 4°C until needed.
6. Place the 132-mm membrane top side up onto a 2XYT AG agar plate and incubate at 37°C for 6 h.
7. Place a second 132-mm nitrocellulose membrane in PBS/T for 30 min. The Tween-20 in PBS/T will block the membrane and prevent proteins from sticking nonspecifically.
8. Remove the second nitrocellulose membrane from the PBS/T and allow to air-dry briefly. Place the dried membrane in a sterile 150-mm Petri dish, and moisten with 2XYT AI.
9. Drain excess 2XYT AI medium from the nitrocellulose membrane (AI membrane). The AI membrane will be used in **step 16**.
10. Weight out 10 mg of silver nanosize-activated powder and place in a sterile 15-mL conical centrifuge tube. Add 1 mL of 0.1 *N* nitric acid. Centrifuge the silver at full speed for 5 min. Decant and discard the nitric acid. Add 1 mL of double-deionized water and centrifuge at full speed for 5 min. Decant and discard the water. Add 1 mL of double-deionized water. Check the pH. If the pH is still acidic, repeat the wash steps with water until the pH is 5.0–8.0.
11. Dilute the silver from **step 11** in 2 mL of PBS to a final concentration of 10 µg/mL. Transfer the silver solution to a sterile Petri dish.
12. Using a pen or pencil, designate a third 132-mm nitrocellulose membrane as a silver-coated membrane.
13. Place the silver-coated membrane face or label-side down onto the silver solution in the Petri dish until moistened. Remove the membrane, invert, and let air-dry.
14. Place the silver-coated membrane in PBS/T for 30 min.
15. Remove the silver-coated membrane and allow to air-dry briefly. Place the silver-coated membrane label side up in a sterile Petri dish, and moisten with 2XYT AI.
16. Drain excess 2XYT AI medium from the silver-coated membrane, and place the AI membrane on top of the silver-coated membrane. Then place the colony membrane top side up (from **step 9**) on top of the AI membrane (*see* **Fig. 1**). Incubate the plate inverted in a humidified container at 30°C for 3 h (*see* **Note 3**).
17. Transfer the colony membrane to a fresh 2XYT AG plate. Store at 4°C. Discard the AI membrane.

18. Wash the silver-coated membrane with PBS/T using three 10-min washes. The silver-coated membrane can be wrapped in a plastic wrap and stored at 4°C overnight, if needed.
19. Dilute HRP/Anti-E tag conjugate 1:8000 in PBS/T. Incubate the silver-coated membrane in this solution for 1 h at room temperature with gentle shaking.
20. Wash the silver-coated membrane with PBS/T using three 10-min washes. Rinse the membrane for 10 s with double-deionized water.
21. Combine equal volumes of chemiluminescent peroxide solution and luminol/enhancer solution according to the manufacturer's instructions.
22. Place the washed membrane into the chemiluminescent substrate for 5 min. Place the silver-coated membrane top side up into a photographic film cassette and cover with plastic wrap. Place the photographic film onto the membrane for varying lengths of time (*see* **Note 4**). Then develop the film. Black areas on the film correspond to bacterial colonies that produce ScFv recombinant antibodies reactive with silver on the membrane (**Fig. 2**).
23. Align the photographic film and colony membrane from **step 18**.
24. Using a sterile pipet tip or toothpick, transfer individual bacterial colonies that give a positive black signal on the film to individual sterile centrifuge tubes containing 500 µL of 2XYT AG with glycerol. Store bacterial isolates as glycerol stocks at –70°C until needed.

3.2. FMAT™ Analysis

1. Add 100 µL of 2XYT AI to each well of a Nunc 384-well plate. This is the replica plate.
2. Dip a 384-pin replicator into the 384-well master plate (*see* **Subheading 3.1., step 3**), remove, and then place into the 384-well replica plate. Incubate the replica plate in a humidified container at 30°C overnight.
3. Centrifuge the replica plate at 500*g* for 10 min. Invert the replica plate over a biohazard bag and flick out the culture supernatant.
4. Using a multichannel pipet, add 40 µL of 1X TES to each well of the 384-well plate followed by 60 µL of 1/5X TES to each well of the plate. Incubate on ice for 1 h.
5. Prepare silver nanosize-activated powder as described in **Subheading 3.1., step 10**.
6. Dilute the silver to 10 µg/mL in PBS/T. Using a multichannel pipet, add 25 µL of diluted silver to each well of a 384-well clear-bottomed black plate (FMAT plate).
7. Using a multichannel pipet, transfer 25 µL of periplasmic extract (prepared in **step 4**) from each well of the 384-well replica plate to the corresponding wells of the FMAT plate.
8. Dilute FMAT Blue Monofunctional Dye/Anti E tag conjugate to 0.75 µg/mL in PBS/T. Using a multichannel pipet, add 25 µL of diluted antibody conjugate to each well of the FMAT plate.
9. Incubate the plate for 2.5 h at room temperature in the dark.
10. Use an FMAT 8100 HTS System to read the microtiter plate. The microtiter plate reader will detect the FMAT Blue Monofunctional Dye/Anti E tag conjugate bound to ScFv recombinant antibodies reactive with silver nanoparticles.

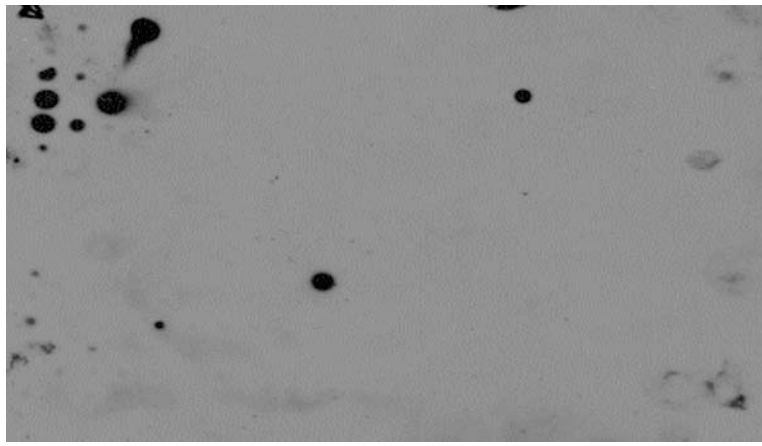

Fig. 2. Photographic film after development of a colony-lifted, silver-coated membrane. Black areas on the film correspond to bacterial colonies that produce ScFv recombinant antibodies reactive with silver on the membrane.

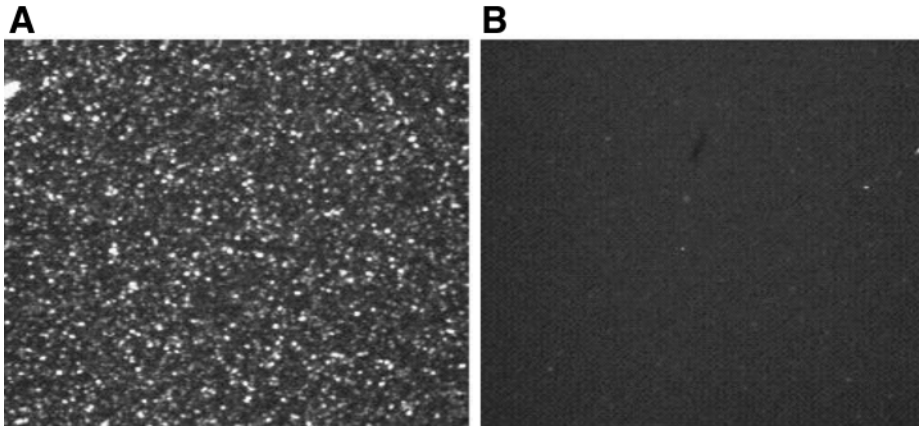

Fig. 3. FMAT results for ScFv on silver nanoparticles: **(A)** detail of ScFv M5, which reacted with silver nanoparticles; **(B)** detail of ScFv G1, which did not react with silver nanoparticles.

11. Based on FMAT analysis results, identify wells from the master plate containing bacterial clones that produce ScFv reactive with silver nanoparticles (**Fig. 3**).
12. Using a micropipet equipped with a sterile pipet tip, transfer 10–30 µL of bacterial culture from positive clones in the master plate to sterile centrifuge tubes containing 500 µL of 2XYT AG with glycerol, mix, and then store at –70°C as glycerol stocks until needed.

4. Notes

1. Multiple dilutions should be performed, since colony numbers will vary from one preparation to another.
2. A humidified container is prepared by lining a sealable plastic container with wet paper towels. Petri dishes, microtiter plates, and so on placed in the container will not dry out during incubation or storage.
3. An 18-gage needle should be used to punch holes through all membrane layers in order to line up the membranes in subsequent steps.
4. Depending on the sensitivity of the substrate and concentration of the ScFv antibodies, the photographic film exposure time will vary. It is best to start with 30 s, but film may need to be exposed for 5 min or longer.

References

1. Storhoff, J. J. and Mirkin, C. A. (1999) Programmed materials synthesis with DNA. *Chem. Rev.* **99,** 1849–1862.
2. Douglas, T. and Young, M. (1999) Virus particles as templates for materials synthesis. *Adv. Mater.* **11,** 679–681.
3. Lee, S. W., Mao, C., Flynn, C. E., and Belcher, A. M. (2002) Ordering of quantum dots using genetically engineered viruses. *Nano. Lett.* **296,** 892–895.
4. Douglas, T., Strable, E., Willits, D., Aitouchen, A., Liberia, M., and Young, M. (2002) Protein engineering of a viral cage for constrained nanomaterials synthesis. *Adv. Mater.* **14,** 415–418.
5. Douglas, T. and Stark, V. (2000) Nanophase cobalt oxyhydroxide mineral synthesized within the protein cage of ferritin. *Inorg. Chem.* **39,** 1828–1830.
6. Mark, A., Willits, D., Mosolf, J., Young M., and Douglas, T. (2002) Protein cage constrained synthesis of ferromagnetic iron oxide nanoparticles. *Adv. Mater.* **14,** 1562–1565.
7. Weiner, S. and Addadi, L. (1997) Design strategies in mineralized biological materials. *J. Mater. Chem.* **7,** 689–702.
8. Spreitzer, G., Whitling, J. M., Madura, J. D., and Wright, D. W. (2000) Peptide encapsulated CdS nanoclusters from a combinatorial ligand library. *J. Chem. Soc. Chem. Commun.* **209,** 210.
9. Whaley, S. R., English, D. S., Hu, E. L., Barbara, P. F., and Belcher, A. M. (2000) Selection of peptides with semiconductor binding specificity for directed nanocrystal assembly. *Nature* **405,** 665–668.
10. Brown, S., Sarikaya, M., and Johnson, E. (2000) A genetic analysis of crystal growth. *J. Mol. Biol.* **299,** 725–735.
11. Rodenburg, C. M., Mernaugh, R. L., Bilbao, G., and Khazaeli, M. B. (1998) Production of a single chain anti-CEA antibody from the hybridoma cell line T84.66 using a modified colony-lift selection procedure to detect antigen-positive ScFv bacterial clones. *Hybridoma* **17,** 1–8.