Isolating recombinant antibodies against specific protein morphologies using atomic force microscopy and phage display technologies

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Isolation of antibodies to antigens that are either unstable, exist in multiple morphologies or have very limited availability can be prohibitively difficult. Here we describe a novel technique combining the capabilities of phage display antibody technology and atomic force microscopy (AFM) that is used to isolate antibody fragments that bind to a specific morphology of the target antigen, α-synuclein. AFM imaging allows us to both visualize the presence and morphology of the target antigen as well as to monitor the efficiency of each step in the bio-panning process. We demonstrate that phage displayed antibodies specific to the target antigen morphology can be isolated after only two rounds of selection. The target antigen, α-synuclein, has been correlated with the Parkinson’s disease (PD). Accumulation of α-synuclein fibrillar aggregates into Lewy body inclusions is a hallmark feature of PD. While α-synuclein can form several different aggregate morphologies including oligomers, protofibrils and fibrils, the role of these morphologies in the progression of PD is not known. The successful selection of the recombinant antibody described here can have potential therapeutic value since the single-chain fragment variable (scFv) can be expressed intracellularly to control folding and toxicity of the specific protein aggregates.

Keywords: antibodies/atomic force microscopy/bio-panning/phage display/protein morphology

Introduction

Phage display technology is a powerful technology that has enabled the rapid generation of recombinant human antibody fragments to a wide variety of antigens (Kristensen and Winter, 1998) and the selection of peptides or proteins with specific binding properties from vast numbers of variants (Willats, 2002). Screening of phage display libraries is usually accomplished by an affinity selection (or bio-panning) process during which phage populations are exposed to the target antigen, non-bound phage are washed off and bound phage are eluted and recovered (Hoogenboom et al., 1998). Successive rounds of bio-panning increases the concentration of phage that bind the target antigen while decreasing the non-specifically bound phage background (Hoogenboom et al., 1998).

Phage display technology has enabled isolation of antibodies to toxic, unstable and non-immunogenic antigens since the process is performed in vitro. Moreover, the amount of target antigen required to isolate antibodies using phage display technology is much less than is typically required when using hybridoma antibody production technology and the time required is usually reduced (Willats, 2002).

Since panning by phage display can be performed in vitro, screening protocols can be readily tailored to the particular requirements of each target molecule. The simplest and most widely used approach is to immobilize target molecules to a support or biotin tag and incubate the antibody library with the immobilized target. Many variations to this theme have been successfully used including fixing the target antigen to coated tubes or probes, within columns or on the surface of magnetic beads (Winter, 1998; Willats, 2002). However, an alternative bio-panning protocol would be very useful for a number of target antigens including those that exist in multiple morphologies, are unstable or are difficult to modify or immobilize.

One set of examples are the proteins involved in neurodegenerative diseases including α-synuclein, β-amyloid, huntingtin and prions, all of which self-assemble into various aggregate morphologies including ring-like structures, thin protofibrils and long fibrils. Isolating antibodies that can bind to specific morphologies of these aggregate forms and/or control the assembly of these different structures would be an extremely valuable tool for studying the role of various morphologies in the progression of the respective diseases.

Here we demonstrate a novel technique of bio-panning using phage display technology combined with atomic force microscopy (AFM) that allows us to isolate antibody fragments that bind specifically to a target morphology of a selected antigen. We utilize a mica surface to immobilize a solution containing the phage display antibody library along with the target antigen (α-synuclein). The negatively charged mica surface will preferentially bind the target antigen, α-synuclein, but not phage particles. We can therefore incubate α-synuclein aggregates with an aliquot of the phage display library, deposit the mixture on mica surface and utilize AFM to image the deposited solution. We can then wash and elute the deposited sample and utilize AFM again to monitor the panning protocol (Figure 1). The target antigen and any complexed phage particles will adhere to the mica surface, while unbound phage can be selectively washed off. Bound phage particles are then eluted off the mica surface and the recovered phage are used to infect the host bacteria and amplified for subsequent rounds of panning. Having both the antigen (α-synuclein) and the complexed phage displayed antibody immobilized on the mica surface has enabled us to monitor each step of the bio-panning process using AFM. In addition, the mica surface allows us to visualize the deposited antigen sample on the surface to verify the target aggregate morphology. Therefore, any desired specific morphology of the antigen can be deposited on mica and its presence verified using AFM. Furthermore, this AFM-based panning technique requires only minimal amounts of antigen...
(nanograms) for the panning process and does not require any modification of the target antigen.

**Materials and methods**

**Materials**

Phage display antibody library  The Tomlinson I and J antibody libraries (MRC Center for Protein Engineering, Cambridge, England) with diversity of $1.47 \times 10^8$ and $1.37 \times 10^8$, respectively, were used for this study. Both libraries are based on a single human framework for $V_H$ and $V_L$. The CDR3 of the heavy chain was designed to be as short as possible yet still maintain an antigen binding surface. Library diversity was achieved by randomization of 11 residues in key CDR2 and CDR3 positions of the heavy chain and 7 residues in the light chain. The libraries contain clones that were pre-selected for active folding domains by binding to Protein-L and Protein-A. Each library was grown separately, and mixed in equal titers for the panning experiments (http://www.hgmp.mrc.ac.uk).

Antigen  Lyophilized $\alpha$-synuclein was a generous gift from Dr V. Uversky (Indiana University School of Medicine). Stock $\alpha$-synuclein was prepared by dissolving the lyophilized powder in phosphate-buffered saline [PBS; 1x (10 mM phosphate and 150 mM NaCl), pH 7.4] and passed through a 0.2 μm filter. Size exclusion chromatography was used to separate monomeric and oligomeric $\alpha$-synuclein forms. Oligomeric $\alpha$-synuclein (>30 kDa) was prepared from lyophilized stock by first dissolving in PBS 1x, pH 7.4, and removing monomeric protein using a 30 kDa filter (Milipore, Billerica, MA). Fibrillar $\alpha$-synuclein was prepared by dissolving the lyophilized stock to a final concentration of 70 μM in buffer containing glycine (0.2 M) and NaCl (150 mM), pH 2.7, then incubating at 56°C for 12 days, followed by 37°C for 7 days, and then stored at 4°C for 10 days. To verify presence of fibrils, an aliquot (10 μl) of the incubated solution was deposited on freshly cleaved mica and fixed for 5 min, washed three times with 1 ml of ultra pure water, dried under a gentle stream of argon gas and imaged by AFM.

**Phage production**  Production of phage from the bacterial library stock was induced by super-infection of bacterial culture with the helper phage KM13 essentially as described previously (http://www.hgmp.mrc.ac.uk). Briefly, bacterial cultures in the log phase ($A_{600} = 0.5–0.6$) were infected with helper phage at a ratio of 1:20 (number of bacterial cells/phage particles) for 30 min at 37°C without shaking. Media was changed to 2x YT containing 100 μg/ml ampicillin (Amp) and 25 μg/ml kanamycin and grown overnight at 30°C for phage production. Phage samples were purified from the supernatant by polyethylene glycol (PEG) precipitation and re-suspended in PBS and used for panning as described below.

Positive control phage sample  We previously isolated an scFv (D10) that binds multiple morphologies of $\alpha$-synuclein (Zhou et al., 2004). This scFv, isolated against full-length $\alpha$-synuclein without regard to morphology, was used as a positive control to develop the bio-panning protocol using AFM. Samples of phage displaying D10 on their surface were purified from the culture supernatant by polyethylene glycol precipitation and re-suspended in PBS. The D10 scFv was used to develop parameters in the AFM panning protocol including phage and antigen concentrations for deposition on the mica surface, incubation times of phage and antigen, deposition times on mica, and wash and elution conditions.

Negative control phage sample  The KM13 helper phage was used as a negative control for developing the AFM panning protocol (Kramer et al., 2003).

**Methods: development of the AFM panning protocol**

Deposition of sample phage  We first determined whether the deposition time of sample on the mica surface would affect the number of bound phage recovered from the mica surface. Initially, an aliquot of $4 \times 10^{12}$ pfu/ml of either the positive or negative control phage samples were incubated with 6 μM mixture of freshly prepared monomeric $\alpha$-synuclein for 2 min. An aliquot (10 μl) of the incubated solution was deposited on freshly cleaved mica and fixed for 5 min. After washing each sample three times with 1 ml ultra pure water, the samples were dried by argon gas and imaged by AFM.
The bound phage was eluted off the mica surface with triethylamine (7.18 M, pH 11) and recovered phage titers were determined for each deposition time for both positive and negative control samples. Different concentrations of both phage and antigen were also tested. Higher concentrations of phage resulted in excessive non-specific binding of the negative control helper phage to the mica surface while higher concentrations of α-synuclein resulted in excessive crowding of aggregates on the mica surface.

Removal of non-bound phage. An aliquot of 4 × 10^{12} pfu/ml of phage from the antibody library was incubated with a 6 μM mixture of freshly prepared α-synuclein for 2 min and a 10 μl aliquot of this solution was deposited on the freshly cleaved mica surface for 5 min. The sample was washed three times with 1 ml of ultra pure water. The sample was then dried under a gentle stream of argon gas and an image was obtained by AFM. The sample was subsequently washed 10 times with 1 ml of 0.1% PBS–Tween and 15 times with PBS in order to eliminate the non-specifically bound phage. The sample was dried and imaged by AFM to verify the presence of bound phage on the mica surface.

**Elution conditions**

We tested seven different elution methods to identify a protocol that would effectively recover bound phage from the mica surface. The elution conditions tested were: (i) incubation with triethylamine (7.18 M, pH 11) for 10 min, followed by neutralization with Tris–HCl (1 M, pH 7.4) and incubation with trypsin (1 mg/ml) for 30 min; (ii) incubation with triethylamine (7.18 M, pH 11) for 10 min, followed by neutralization with Tris–HCl (1 M, pH 7.4); (iii) trypsin alone (1 mg/ml); (iv) trypsin alone (1 mg/ml) with CaCl₂ (1 mM); (v) glycine buffer (50 mM, pH 2.2), followed by neutralization with Tris buffer (1 M, pH 8); (vi) glycine buffer (50 mM, pH 2.2), followed by neutralization with Tris buffer (1 M, pH 8); and subsequently adding CaCl₂ (1 mM) and trypsin (1 mg/ml) and incubation for 15 min (vii) adding triethylamine (7.18 M, pH 11) and incubation for 10 min, neutralization with 1 M Tris–HCl, pH 7.4, and followed by a 30 min incubation with trypsin (1 mg/ml) and CaCl₂ (1 mM). Eluted phage was titered and amplified as described. AFM images were obtained from each mica surface after elution using each of the seven different elution protocols.

**AFM panning against fibrillar α-synuclein**

Selection of phage against the fibrillar morphology of α-synuclein was performed by incubating an aliquot of 4 × 10^{12} pfu/ml of the phage obtained from the initial antibody library with a 4 μM sample of fibrillar α-synuclein for 2 min. Presence of fibrillar α-synuclein was verified by AFM. A 10 μl aliquot of the incubated solution was deposited on freshly cleaved mica and fixed for 5 min and then the substrate was washed three times with 1 ml of ultra pure water. The sample was then dried under a gentle stream of argon gas and an image was obtained by AFM. The sample was subsequently washed 10 times with 1 ml of 0.1% PBS–Tween and 15 times with PBS in order to eliminate the non-specifically bound phage. After several rounds of washing, the sample was dried and imaged by AFM to verify the presence of bound phage on the mica surface. The bound phage was eluted from the mica surface by incubation with triethylamine (7.18 M, pH 11) for 10 min, followed by neutralization with 1 M Tris–HCl, pH 7.4, and then hydrolysis with trypsin (1 mg/ml) and Calcium (1 mM) for 30 min. To determine the number of eluted phage, 1.2 ml of TG1 at OD_{600} = 0.4 was added to the 500 μl of eluted phage and incubated for 30 min at 37°C. The infected *Escherichia coli* TG1 cells were plated in serial dilution on agar containing ampicillin (100 μg/ml) plates. Eluted phage was amplified by infection of fresh *E.coli* TG1 cells grown to an OD = 0.4 at 600 nm in the presence of helper phage KM13 (5 × 10^{10}) according to standard protocols (http://www.hgmp.mrc.ac.uk). The phage was purified from the culture supernatant by polyethylene glycol precipitation and re-suspended in PBS and used for the next round of panning as described above.

**Selection by phage enzyme-linked immunosorbent assay (ELISA)**

**Polyclonal ELISA** High binding microtiter plates (Corning, USA) were coated overnight at 4°C with 50 μg/ml human recombinant fibrillar α-synuclein in carbonate-bicarbonate (50 mM, pH 9.6). Non-specific binding was blocked by incubation with 3% bovine serum albumin (BSA) for 2 h at 37°C. A 10^{10} titer unit aliquot of PEG precipitated phage in 100 μl of 2% BSA–PBS was added to the wells and incubated for 90 min at room temperature. Bound phage was detected after a 1-h incubation with a 1:5000 dilution of anti-M13 antibody horse-radish peroxidase (HRP) conjugate. A 100 μl aliquot of the HRP substrate 3,3',5,5'-tetrarmethylbenzidine (TMB, Sigma) was added and the reaction stopped after 20 min with 2 M H₂SO₄ (VWR Scientific Products, PA). Activity was determined by subtracting OD_{450} from OD_{650} using a Wallac 1420 plate reader (Perkin Elmer).

**Monoclonal ELISA** Individual clones obtained from fibrillar panning were grown essentially as described (http://www.mrc.cpe.cam.ac.uk). The high binding microtiter plates were coated overnight at 4°C with 50 μg/ml of human recombinant fibrillar α-synuclein in carbonate-bicarbonate buffer (50 mM, pH 9.6). Non-specific binding was blocked by incubation with PBS + 2% non-fat milk (2% PBSM) for 2 h at room temperature. Bacterial supernatant containing antibody fragments was added to each well (100 μl/well). Bound phage was detected after 1-h incubation as described above.

**Production and purification of scFv**

Soluble scFv was produced by expressing recovered phagemid samples in the non-suppressor *E.coli* strain HB2151 essentially as described previously (http://www.hgmp.mrc.ac.uk). Individually selected clones were grown and scFv induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated overnight at 4°C with 50 μg/ml of human recombinant fibrillar α-synuclein in carbonate-bicarbonate buffer (50 mM, pH 9.6). Non-specific binding was blocked by incubation with PBS + 2% non-fat milk (2% PBSM) for 2 h at room temperature. Bacterial supernatant containing antibody fragments was added to each well (100 μl/well). Bound phage was detected after 1-h incubation as described above.
bound scFv was eluted from column with 0.2 M glycine, pH 3. Fractions containing scFv were pooled, adjusted to neutral pH, dialysed and stored at −20°C. The purity of the scFv was estimated by electrophoresis on 12% (W/V) SDS–polyacrylamide gel.

**Soluble scFv ELISA** Both supernatant and periplasmic fractions were assayed for antigen binding by ELISA as described above. High affinity microtiter plates were coated with 50 μg/ml of human recombinant fibrillar α-synuclein. After blocking and washing, the supernatant or periplasmic fraction containing antibody fragments was added to each well (100 μl/well) and the plate was incubated for 2 h at room temperature. Plates were washed three times with PBS + 0.1% Tween-20 (PBST). Anti-myc 9E10 antibody was added to the wells at a dilution of 1:500 and plates were incubated for 1 h. Plates were washed three times with PBST. Bound antibodies were detected after a 1-h incubation using a 1:1000 dilution of goat anti-mouse IgG HRP conjugated.

**Plasmid preparation and PCR amplification**
Plasmid was isolated from *E. coli* TG1 using a Qiagen Plasmid Miniprep Kit (QIAGEN, Valencia, CA) according to the manufacturer’s protocols. The presence of a full-length scFv insert was confirmed by PCR using 24 cycles of amplification with the phagemid DNA as template and the forward primer, pHEN seq (5'-CTATGGGCCCCTTCA) and the backward primer, LMB3 (5'-CAGGAAACAGCTATGAC). PCR analysis was performed using 1.5% (w/v) agarose gels (Merck).

**AFM imaging**
Mica was used as an AFM substrate. A 10 μl aliquot of each sample was placed on mica for 5 min and rinsed three times with 1 ml of ultra pure water. The sample was then dried under a gentle stream of argon gas and an image was obtained by AFM. The sample was subsequently washed 10 times with 1 ml PBS–Tween (0.1%) and 15 times with PBS in panning with phage from the initial phage library. The sample was then dried under a gentle stream of argon gas and an AFM image was obtained. All images were acquired in air using a tapping mode AFM with NanoScope IIIa controller (Veeco/ Digital Instruments, Santa Barbara, CA). The cantilevers

![AFM image of phage on mica surface. (A) Positive D10 phage control shown binding to α-synuclein aggregates. (B) Negative helper phage control does not bind α-synuclein aggregates. Thin filaments in (A) represent phage particles (height 1.7–1.9 nm). Aggregates of α-synuclein are represented by various size round white images.](image1)

![AFM image of fibrillar α-synuclein. (Height 4–6 nm).](image2)

**Results**

*Developing bio-panning protocol using AFM*
Using the previously isolated human single-chain Fv (scFv) antibody D10, which binds multiple morphologies of α-synuclein, as a positive control (Zhou *et al.*, 2004), and KM13 helper phage lacking any displayed antibody as a negative control, we determined the antibody and antigen concentrations for co-incubation, deposition time, and wash conditions necessary to utilize AFM as a panning tool.

**Deposition time**
Recovery of positive phage samples from the mica surface increases only slightly with deposition times ranging from 2 to 10 min, while recovered phage titers of the negative control
 increases substantially with deposition times >5 min (data not shown). AFM images verify the presence of the positive control phage (D10) (Figure 2A) and the absence of the negative control helper phage (Figure 2B) on the mica surface for the 5-min deposition samples.

**Elution conditions**

Of the seven different elution protocols tested, treatment with triethylamine (TEA) and trypsin yielded the highest recovery of phage (Table I). Treatment with trypsin cleaves the pIII protein in the helper phage KM13 used in this study, rendering phage that do not express an scFv non-infective (Kristensen and Winter, 1998). Recovery of phage from the mica surface using the TEA/trypsin elution protocol was verified by AFM imaging, indicating only very few bound phage remaining on the mica surface (data not shown).

**AFM bio-panning against fibrillar α-synuclein**

After developing suitable deposition, wash and elution protocols that enable us to bio-pan directly on a mica substrate, we tested the protocol by panning for antibodies to the fibrillar morphology of α-synuclein using the initial antibody library. After verifying the presence of fibrillar α-synuclein by AFM (Figure 3), we performed three rounds of panning against the synuclein fibrils using the antibody library. After only two rounds of panning however, most of the phage remaining after the wash step was bound to synuclein aggregates as indicated by AFM analysis (Figure 4).

**Quantification of positive binding phase**

After three rounds of selection, 22 of 48 randomly selected clones isolated from the third round elution sample showed positive binding (values >5× the SD) to fibrillar α-synuclein as indicated by phage ELISA (Table II). Twelve randomly selected positive clones all contained full-length scFv’s as indicated by PCR (data not shown). We then determined the binding specificity of three of the full-length scFvs (6E, 4B and 3E) toward four different morphologies of α-synuclein by monoclonal phage ELISA. One scFv (6E) bound only to fibrillar α-synuclein, one (4B) bound only to the monomer and fibrillar, and one (3E) bound only to large oligomeric and fibrillar forms (Table III). While all the clones have interesting binding properties, we initially selected 6E for further study based on its specificity for the fibril morphology.

**DNA sequence of 6E**

DNA sequencing revealed the presence of a spurious stop codon (TGA) in a constant region of the 6E scFv (data not shown). The stop codon was replaced with a tryptophan (TGG)
**Expression and purification of soluble scFv**

We purified soluble scFv from the corrected 6E clone for further characterization. Production of soluble scFv in the non-suppressor E.coli strain HB2151 showed a 29 kDa band corresponding to the expression size of a full-length scFv (data not shown). The scFv specifically recognized the fibrillar form of α-synuclein but not the monomeric and oligomeric form as determined by soluble ELISA (Table IV).

**Table IV.** Soluble ELISA of 6E clone against α-synuclein

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Control</th>
<th>Monomer</th>
<th>Oligomer</th>
<th>Large oligomer</th>
<th>Fibrillar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.000 ± 0.002</td>
<td>1.099 ± 0.004</td>
<td>0.931 ± 0.002</td>
<td>1.000 ± 0.004</td>
<td>1.021 ± 0.005</td>
</tr>
<tr>
<td>6E</td>
<td>1.000 ± 0.029</td>
<td>1.047 ± 0.037</td>
<td>0.740 ± 0.003</td>
<td>1.213 ± 0.086</td>
<td>1.932 ± 0.055</td>
</tr>
</tbody>
</table>

ELISA showing binding of purified control scFv (anti-phosphorylase B) and 6E to four different morphologies of α-synuclein: monomeric, oligomeric, large oligomer (≥30) and fibrillar. Activity was determined by subtracting OD_scFv from OD_α-syn and comparing absorbance between controls well (without immobilized α-synuclein) to the sample well (with α-synuclein) where the control value was normalized to 1. The data represent the average ± standard deviation (SD) of two experiments with experimental value being repeated in triplicate.

Discussion

Phage display technology is a powerful method for the rapid generation of specific recombinant human antibody fragments (Kristensen and Winter, 1998). The simplest and most widely used approaches are to immobilize target molecules to a support or bind them to biotin and then to expose solutions containing phage to the immobilized target (Kristensen and Winter, 1998; Willats, 2002) or recover the bound phage complexes using streptavidin beads (Pini et al., 1998; Ansuini et al., 2002). Numerous variations to this theme have been successfully employed. In this study we utilize mica, a frequently used substrate in AFM, to immobilize the bound antigen/antibody complex. Using a novel bio-panning protocol, we incubated solutions containing phage and our target antigen (fibrillar α-synuclein) and deposited them on the mica surface and isolated antibody fragments that bound to our target α-synuclein morphology. The non-binding phage was removed from the surface and bound phage eluted and amplified for subsequent rounds of panning. Having both the antigen (α-synuclein) and the phage displayed antibody immobilized on the mica surface has enabled us to monitor each step of the bio-panning process using AFM. The ability to visualize various steps of bio-panning via AFM has made it possible for us to determine the efficiency of each step of the bio-panning process, helping to increase selection of positive phage and decrease recovery of non-specifically bound phage. By monitoring the bio-panning process by AFM we were able to verify both that, the specific antigen morphology we were targeting was present and also that each step of the bio-panning protocol was working efficiently.

We demonstrated that 22 of 48 clones (46%) isolated after three rounds of selection against fibrillar α-synuclein had bound to the target antigen. The use of mica as a substrate gives us the potentially very useful advantage of being able to visualize the deposited antigen sample on the surface without having to chemically modify it. For antigens that exist in multiple forms or that have unstable intermediates, this protocol provides a powerful tool to isolate scFv’s that bind to the specific target morphology. Furthermore, this protocol utilizes only very small amounts of antigen (nanograms) during the panning process and may be very useful for isolating antibodies to antigens of limited availability or stability. In addition, this method can be used as a generalized protocol for phage selection of the recombinant antibody expressed on the surface of bacteriophage by this approach can have potential therapeutic value once this scFv is expressed intracellularly in order to control folding of protein aggregates.

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References


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